REMARKS

Claims 12-15, 18, 20, 21, and 56-58 are currently pending in this application. Claim 58 is withdrawn from consideration. Claims 12-15, 18, and 20 are rejected under 35 U.S.C. § 112, first paragraph, for lack of written description. Claims 12-15, 18, 20, 21, 56, and 57 are rejected under 35 U.S.C. § 112, first paragraph, for new matter. Claims 12-15, 18, 20, 21, 56, and 57 are rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. By this reply, Applicant adds new claim 59, amends claims 12 and 21, cancels claim 20, and addresses each of the rejections below.

Support for the Amendment

Support for the amendment to claim 12 is found in prior claim 20. Claim 21 is amended to correct claim dependency. Support for new claim 59 is found in the specification at, e.g., page 18, lines 10-15, and page 49, lines 5-7. No new matter is added by the amendment.

Interview with Examiner Juedes

Inventor Faustman and Applicant's representatives wish to thank Examiner Juedes for her participation in an in-person interview on November 19, 2007. The written description and enablement rejections of claims 12-15, 18, and 20-21 were discussed. During the interview, Examiner Juedes acknowledged that additional data presented by the inventor, Dr. Faustman, during the interview would likely overcome the enablement rejection, but requested that Applicant provide further evidence in response to the written description rejection. As requested, Applicant provides the requested information, which is discussed in more detail below.

Applicant believes that present claims 12-15, 18, 21, 56, 57, and 59 are in condition for allowance, and Applicant respectfully requests the mailing of a notice to that effect. If the Office does not agree, Applicant respectfully requests that the Office contact the undersigned by telephone in order to resolve any remaining issues in this case.

Rejections under 35 U.S.C. § 112, first paragraph

Written Description

The Office rejects claims 12-15, 18, and 20 under 35 U.S.C. § 112, first paragraph, for lack of written description, stating "there is insufficient written description to demonstrate that applicant was in possession of the claimed genus of 'TNF-alpha agonists' or 'TNF-alpha inducing substances'" (Office Action, p. 6). Applicant respectfully disagrees with the Office's conclusion, but in the interest of expediting prosecution of the present application, Applicant has amended present independent claim 12 to recite the use of "TNF-alpha receptor agonists" and to remove "TNF-alpha inducing substances" from the claim. Applicant reserves the right to pursue cancelled subject matter in a later filed application that claims benefit to the present application.

The M.P.E.P. § 2164.05(a) states:

> The specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

Present independent claim 12, and claims dependent therefrom, recite the use of, e.g., a genus of TNF-α receptor agonists that preferentially decrease the viability of leukocytes for diagnosing an autoimmune disease in a mammal or for determining a mammal's predisposition to develop an autoimmune disease. The Office states that Applicant's specification only discloses antibody agonists of TNF-α, which "is not representative of the broad range of structurally different agonists encompassed by the claims" (Office Action, p. 7). Applicant respectfully disagrees.

It is unnecessary for Applicant to describe the genus of TNF-α receptor agonists in any more detail than that already provided in the specification because TNF-α receptor agonists are already well known to the skilled artisan. In particular, TNF-α receptor agonists, such as TNF-α muteins, TNF-α agonist antibodies, TNF-α agonist peptides, and TNF-α agonist compounds, were all known in the art at the time of Applicant's priority date. For example, each of U.S. Patent Nos. 5,486,463 and 5,422,104; PCT Publication Nos. WO 86/02381; WO 86/04606; and

WO 88/06625; and European Patent Nos. 155,549; 168,214; 251,037; 340,333; and 486,908 disclose TNF-α muteins capable of acting as TNF-α receptor agonists. In addition, TNF-α receptor agonist antibodies that could be used in the method of present independent claim 12, and claims dependent therefrom, are disclosed in, e.g., Galloway et al. (Eur. J. Immunol. 22:3045-3048, 1992), Tartaglia et al. (J. Biol. Chem. 268:18542-18548, 1993), Tartaglia et al. (J. Immunol. 151:4637-4641, 1993), Smith et al. (J. Biol. Chem. 269:9898-9905, 1994), and Amrani et al. (Am. J. Respir. Cell. Mol. Biol. 15:55-63, 1996). TNF-α receptor agonist peptides are disclosed in, e.g., Pontzer et al. (BBAC 193:1191-1197, 1993), Balazovich et al. (Blood 88:690-696, 1996), Kranzhöfer et al. (Circ. Res. 79:286-294, 1996), Hoffman et al. (Blood Cells Mol. Dis. 21:156-167, 1995), and Kumaratilake et al. (J. Clin. Invest. 95:2315-2323, 1995). Finally, TNF-α receptor agonist compounds are described in, e.g., Strassman et al. (Cell Immunol. 176:180-185, 1997), Perera et al. (Infect. Immunol. 61:2015-2023, 1993), Rose et al. (J. Immunol. 144:3513-3517, 1990), Cembrzyńska-Nowak et al. (J. Interferon Cytokine Res. 17:609-617, 1997), Boland et al. (J. Biol. Chem. 272:12952-12960, 1997), Das et al. (J. Biol. Chem. 272:14914, 1997), and Dirsch et al. (Mol. Pharmacol. 53:402-407, 1998). Copies of the abstracts of the cited non-patent publications are provided.

As is clear from the publications mentioned above, the genus of TNF-α receptor agonists was well known prior to Applicant's invention. Accordingly, the metes and bounds of this genus would have been clearly understood by the skilled artisan at the time the application was filed upon reading Applicant's specification. Therefore, contrary to the Office's position, Applicant's specification clearly conveys to the skilled artisan that the inventor was in possession of the full scope of the claimed invention at the time of filing and provides considerable guidance that would allow the skilled artisan to carry out the full breadth of the claimed invention. For this reason, present claims 12-15 and 18 comply with the requirements of 35 U.S.C. § 112, first paragraph, and withdrawal of this rejection is respectfully requested.

New Matter

The Office rejects claims 12-15, 18, 20, 21, 56, and 57 under 35 U.S.C. § 112, first paragraph, for new matter. In particular, the Office states that the specification lacks support for

the term "TNF-alpha agonist" and for the phrase "a statistically significant decrease" recited in independent claim 12. As is discussed above, present independent claim 12 now recites the use of a "TNF-alpha *receptor* agonist," which the Office acknowledges is supported by the present specification (see, e.g., Office Action, p. 8). The rejection as it applies to this term should be withdrawn.

With respect to the phase "a statistically significant decrease," the Office states:

> [T]he instant specification discloses measuring a decrease of leukocyte viability. However, the only disclosure of a "statistically significant" decrease is found in the specific examples, which involve measuring TNF-alpha induced T cell death in patients with type I diabetes. This has a much narrower scope than the instant claims, which encompass measuring a "statistically significant" decrease in viability after contact with an TNF-alpha inducing substance or agonist in a sample from a mammal with any autoimmune disease. (Office Action, p. 8.)

Applicant respectfully traverses this rejection.

The specification teaches an assay method for diagnosing many different autoimmune diseases in a mammal (see the list on, e.g., page 33, line 12, through page 34, line 6). Although the specification exemplifies the assay method using leukocytes from patients having type I diabetes, as is clear from the title of Example 2, the assay method applies to the diagnosis of "autoimmune disease," not just type I diabetes. Thus, although Example 2 of Applicant's specification exemplifies the assay method using peripheral blood lymphocytes from human diabetic patients and NOD mice, the determination of a statistically significant decrease in leukocyte viability when performing the presently claimed assay method is an aspect of the method that applies to the determination of any autoimmune disease and is not unique to the diagnosis of type I diabetes.

This is further apparent on, e.g., page 50, which discusses an automated assay method for detecting cell death using high-throughput cellular screening, which "enables the rapid, *quantitative analysis* of small amounts of human blood to measure the phenotype penetrance of heightened apoptosis" (Specification, p. 51, lines 16-18; emphasis added). The specification states that "[f]low cytometric analysis is useful as a reliable assay for the detection of heightened TNF-α sensitivity (e.g., TNF-alpha induced cell death) in *autoimmune patients (e.g., human diabetics)*" (Specification, p. 50, lines 14-16; emphasis added). Thus, the assay method applies

broadly to the diagnosis of autoimmune diseases generally, not just type I diabetes.

Because one of skill in the art, upon reading Applicant's specification, would understand that the measuring of "a statistically significant decrease" in leukocyte viability applies generally to the diagnosis of autoimmune diseases and is not unique to the diagnosis of type I diabetes, the rejection of independent claim 12, and claims dependent therefrom, for new matter in view of the phrase "a statistically significant decrease" should be withdrawn.

Enablement

The Office rejects claims 12-15, 18, 20, 21, 56, and 57 under 35 U.S.C. § 112, first paragraph, for lack of enablement, stating that "the specification provides insufficient evidence that the claimed method would function to diagnose autoimmune disease as broadly claimed" (Office Action, p. 3). Applicant respectfully traverses this rejection.

The Office acknowledges that "[t]he data in the instant specification demonstrates that leukocytes from subjects with type I diabetes are more susceptible to TNF-alpha mediated cell death," but states that "[t]his clearly does not bear a reasonable correlation to a method encompassing diagnosing any autoimmune disease with any TNF-alpha inducing substance or any TNF-alpha agonist" (Office Action, p. 5). Applicant respectfully disagrees. Applicant submits publications authored by the inventor, at least one of which includes data, which were discussed during the in-person interview of November 19, 2007, that support the enablement of present claims 12-15, 18, 21, 56, 57, and 59.

I. Applicant's *in vitro* and *in vivo* Data Support the Enablement of Present Claims 12-15, 18, 21, 56, 57, and 59

Applicant has demonstrated that NFκB precursor proteins are not processed properly in the NOD mouse resulting ineffective signaling within the NFκB pathway after TNF-α exposure and failure of anti-apoptotic gene expression. Applicant has discovered that the ineffective signaling within the NFκB pathway is at least partially responsible for the susceptibility of autoreactive T-cells to killing by TNF-α and TNF-α receptor agonists (e.g., TNF-α receptor agonist antibodies). Using *in vitro* and *in vivo* assays, Applicant has demonstrated that TNF-α and TNF-α receptor agonists specifically eliminate autoreactive T cells responsible for

autoimmune disease. Through a series of experiments, discussed in detail below and in the attached publications, Applicant has shown that the method of present claims 12-15, 18, 21, 56, 57, and 59 is enabled to diagnose autoimmune disease.

Agonism of the TNF-α Pathway Kills Auto-Reactive CD8+ T cells (CTLs) but not CD4+ T cells

Applicant's specification teaches the use of TNF-α and TNF-α receptor agonists, which stimulate the TNF-alpha signaling pathway, cause killing of the autoreactive immune cells involved in autoimmune disease, the detection of which can be used to diagnose autoimmune disease in a mammal. Applicant has shown that agonism of the TNF-α pathway targets a specific subset of autoreactive CD8+ T cells that are responsible for mediating human autoimmune disease, as is discussed below.

In Kodama et al. (Cell. Mol. Life Sci. 62:1850-1862, 2005, a copy of which is provided herewith), Applicant confirmed that *in vitro* stimulation of the TNF-α pathway in cells from NOD mice specifically eliminates autoreactive CD8+ T cells (see, e.g., Figure 3). Detection of the death of these autoreactive CD8+ T cells following *in vitro* stimulation of the TNF-α pathway confirms not only their presence in the animal, but also the presence of autoimmune disease in the animal. Conversely, the absence of evidence of CD8+ T cell death following *in vitro* stimulation of the TNF-α pathway in cells taken from the animal correlates with the absence of autoimmune disease in the animal.

This conclusion is reaffirmed in Kühtreiber et al. (J. Immunol. Methods 306:137-150, 2005, a copy of which is provided herewith), in which Applicant, and colleagues working under her direction, isolated splenocytes from diabetes-prone NOD and control mice and identified a quantifiable subpopulation of T cells, with co-expression of CD8, that selectively undergo cell death on exposure to TNF-α (see Results, §§ 3.3-3.5, pages 142-146; Kühtreiber et al., *supra*). As is clearly disclosed in Kühtreiber et al., only the subset of autoreactive CD8+ T cells undergo cell death in response to TNF-α receptor agonism (see, e.g., page 146, col. 1).

Moreover, Applicant determined that this subpopulation of autoreactive splenic CD8+ T cells is only present in NOD mice that develop disease; autoreactive splenic CD8+ T cells are absent in NOD mice that do not develop disease. Applicant exposed splenic CD8+ T cells from

NOD mice that develop diabetes and invasive insulinitis to a TNF-α receptor agonist and observed the death of these cells, whereas the exposure of splenic T cells from NOD mice that never become diabetic and only develop peripheral, not invasive, insulinitis, which is a hallmark of nonprogression of autoimmune disease, to a TNF-α receptor agonist does not result in cell death (see page 146, § 3.6; Kühtreiber et al., *supra*). Thus, Applicant's data from the studies of NOD mice, which are an accepted animal model of type 1 (autoimmune) diabetes mellitus, Sjogren's syndrome, and lupus in humans, confirm the presence of a specific subpopulation of autoreactive T cells that are sensitive to TNF-α receptor agonists, which promote the death of these cells. Applicant's *in vitro* and *in vivo* mouse data show that mammals that develop autoimmune disease uniquely possess these autoreactive CD8+ T cells and that these cells specifically die in response to TNF-α receptor agonism, all of which support the enablement of present claims 12-15, 18, 21, 56, 57, and 59.

II. Successful *in vitro* Results Using Human Cells Confirms that the Results in the NOD Mouse Model is Predictive of Success in Diagnosing Autoimmune Disease in Humans

Applicant has conducted further experiments with cells from human patients diagnosed with one of several different autoimmune diseases and have confirmed that the results in NOD mice discussed above also occur in humans. Namely, TNF-α and TNF-α receptor agonists that stimulate the TNF-α signaling pathway bring about the killing of a subpopulation of autoreactive CD8+T cells involved in autoimmune disease in humans. The sensitivity of these CD8+T cells to cell killing by exposure to TNF-α and TNF-α receptor agonists can be used to diagnose multiple different and diverse autoimmune diseases in humans.

Applicant's data shows that peripheral blood lymphocytes taken from human patients diagnosed with one of several different autoimmune diseases, e.g., type I diabetes, lupus, psoriasis, Crohn's disease, Graves' disease, Sjögren's syndrome, hypothyroidism, celiac disease, rheumatoid arthritis, and multiple sclerosis, and exposed *in vitro* to TNF-α and TNF-α receptor agonists undergo apoptosis, but that cells taken from humans who are not diagnosed with autoimmune disease do not undergo apotosis (see, e.g., Figs. 1-4 and SI Fig. 2 of Ban et al.,

manuscript submitted for publication (2008), a copy of which is provided).

Thus, Applicant's data show that the method of present claims 12-15, 18, 21, 56, 57, and 59 can be used to diagnose autoimmune disease (or a predisposition to develop autoimmune disease) by measuring the viability of leukocytes obtained from a mammal to be tested following their contact with TNF-α or a TNF-α receptor agonist and detecting a statistically significant decrease in cell viability relative to a second, control sample containing leukocytes (e.g., from a normal mammal). Accordingly, the full scope of the method of present claims 12-15, 18, 21, 56, 57, and 59 is predictable and enabled.

For this reason, Applicant respectfully requests that the rejection of claims 12-15, 18, 21, 56, 57, and 59 under 35 U.S.C. § 112, first paragraph, for lack of enablement be withdrawn.

CONCLUSION

In view of the above remarks, Applicant respectfully submits that the claims are in condition for allowance, and such action is respectfully requested.

Enclosed is a Petition to extend the period for replying to the Office Action for three months, to and including July 8, 2008, and authorization to charge the fee required by 37 C.F.R. § 1.17(a) to Deposit Account No. 03-2095.

If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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Date: 2 July 2008

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1: Eur J Immunol. 1992 Nov;22(11):3045-8.

Links

Anti-tumor necrosis factor receptor and tumor necrosis factor agonist activity by an anti-idiotypic antibody.

Galloway CJ, Madanat MS, Sarr T, Espevik T, Dumas ML, Mitra G, Ranges GE.

Miles, Incorporated, (Inc.), Berkeley, Miles, Inc., West Haven.

Tumor necrosis factor (TNF) is a cytokine which, among other properties, is a principle mediator of inflammation and septic shock. It acts upon target cells by binding to specific cell surface receptors. A10G10 is a murine monoclonal antibody which recognizes human TNF and neutralizes its activity. A rabbit polyclonal antibody directed at the antigen-binding site of A10G10 was raised and affinity purified over an A10G10 column. The resultant anti-idiotypic antibody recognized not only A10G10 but also both TNF receptors. It showed TNF agonist activity in two different TNF bioassays, and competed with several anti-TNF receptor monoclonal antibodies and TNF itself for binding to cells. These results represent an example of a method for obtaining antibodies to a ligand-specific receptor in the absence of the receptor itself.

PMID: 1330580 [PubMed - indexed for MEDLINE]

1: J Biol Chem. 1993 Sep 5;268(25):18542-8.

Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor.

Tartaglia LA, **Pennica D**, **Goeddel DV**.

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To understand the role of the 75-kDa tumor necrosis factor (TNF) receptor in non-lymphoid cells, the cytotoxic signaling and ligand binding activities of the 55-kDa (TNF-R1) and 75-kDa (TNF-R2) TNF receptors were investigated using agonist and antagonist antibodies specific for the two receptor types. This study indicates that although TNF-R2 can significantly reduce the TNF concentration required for cell killing, the mechanism by which this is accomplished is not through the generation of an intracellular signal by TNF-R2. Instead, TNF-R2 regulates the rate of TNF association with TNF-R1, possibly by increasing the local concentration of TNF at the cell surface through rapid ligand association and dissociation. We propose that other cell-surface receptors, such as the low affinity p75 nerve growth factor receptor, may utilize an analogous "ligand passing" mechanism.

PMID: 8395508 [PubMed - indexed for MEDLINE]

1: J Immunol. 1993 Nov 1;151(9):4637-41.

Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor.

Tartaglia LA, **Goeddel DV**, **Reynolds C**, **Figari IS**, **Weber RF**, **Fendly BM**, **Palladino MA Jr**.

Department of Molecular Biology, Genentech, Inc., South San Francisco, CA 94080.

TNF-alpha can enhance the proliferation of human thymocytes stimulated by the comitogen Con A. To determine which of the two different TNF receptors is responsible for signaling this cellular response, we investigated the proliferation of human thymocytes in response to agonistic antibodies specific for the two TNF receptor types. In contrast to previously examined TNF activities in human cells, thymocyte proliferation was stimulated in response to rabbit polyclonal antibodies directed against the 75-kDa TNF receptor (TNF-R2), but not those directed against the 55-kDa TNF receptor (TNF-R1). Lymphotoxin (TNF-beta) was also shown to stimulate human thymocyte proliferation, demonstrating that TNF-beta can initiate a biologic response that is mediated by TNF-R2. TNF-R2-mediated T-cell proliferation was not restricted to the immature T cells within the thymus, as the anti-TNF-R2 antibodies also stimulated the proliferation of peripheral T cells. As a first step toward identifying a specific agonist of TNF-R2 with therapeutic potential, 10 anti-TNF-R2 mAb were examined for potential agonist activity. Nine of these significantly stimulated human thymocyte proliferation with maximal responses ranging from twofold to significantly greater than that obtained with TNF-alpha by itself.

PMID: 8409424 [PubMed - indexed for MEDLINE]

1: J Biol Chem. 1994 Apr 1;269(13):9898-905.

Enhanced synthesis of tumor necrosis factor-inducible proteins, plasminogen activator inhibitor-2, manganese superoxide dismutase, and protein 28/5.6, is selectively triggered by the 55-kDa tumor necrosis factor receptor in human melanoma cells.

Smith DM, **Tran HM**, **Soo VW**, **McQuiston SA**, **Tartaglia LA**, **Goeddel DV**, **Epstein LB**.

Cancer Research Institute, University of California, San Francisco 94143-0128.

We have demonstrated that A375 melanoma cells express mRNA for both types of tumor necrosis factor (TNF) receptors and receptor proteins on their plasma membranes. Specific agonist and blocking antibodies to either 55-kDa (TNF-R1) or 75-kDa (TNF-R2) TNF receptors combined with two-dimensional gel analysis were employed to determine which receptor type is responsible for mediating the induction of individual melanoma proteins. Our results indicate that the enhanced synthesis of proteins 21/>7 (M(r)/pI), 28/5.6, and 41/5.7 is selectively induced through TNF-R1. TNF induces these proteins; antagonist antibody to TNF-R1 prevents their induction by TNF, and TNF-R1 agonist induces them in the absence of TNF. Identification of these proteins by immunoblot analysis proved that 21/>7 is manganese superoxide dismutase, protein 28/5.6 is unrelated to 27/28-kDa heat shock protein, and protein 41/5.7 is plasminogen activator inhibitor-2. Furthermore, TNF cytotoxicity for A375 cells is also mediated by TNF-R1. These studies indicate that TNF-R1 is a critical signaling receptor for TNF action on A375 cells and demonstrate the potential use of TNF-R1 antibodies to selectively block or enhance specific effects of TNF on melanoma cells.

PMID: 8144583 [PubMed - indexed for MEDLINE]

1: Am J Respir Cell Mol Biol. 1996 Jul;15(1):55-63.

Links

Activation of the TNF alpha-p55 receptor induces myocyte proliferation and modulates agonist-evoked calcium transients in cultured human tracheal smooth muscle cells.

Amrani Y, Panettieri RA Jr, Frossard N, Bronner C.

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Evidence suggests that cytokines may modulate smooth muscle cell function in a variety of inflammatory diseases. In the present study, we characterized the specific receptor subtypes that mediate tumor necrosis factor alpha (TNF alpha) effects on myocyte proliferation and on agonist-induced calcium transients in cultured human tracheal smooth muscle cells (TSMC). Pretreatment of human TSMC with TNF alpha potentiated cytosolic calcium [(Ca2+)i] transients evoked by carbachol. In a similar manner, selective TNF alpha-p55 receptor agonists such as htr-9, an activating monoclonal antibody, or a recombinant TNF-p55 (rTNF-p55), which specifically activates the TNF alpha-p55 receptor but not the TNF alpha-p75 receptor, also augmented [Ca2+]i transients evoked by carbachol. In parallel experiments, TNF alpha, rTNF alpha-p55, and htr-9 induced human TSMC proliferation as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Interestingly, activation of the TNF alpha-p75 receptor with a selective agonist, recombinant TNF alpha-p75 (rTNF alpha-p75), or inhibition of the TNF alpha-p75 receptor with utr-1, an inhibitory anti-TNF alpha-p75 receptor antibody, had no effect on TNF alpha-augmented calcium transients or on myocyte growth. To further confirm the receptor specificity of these findings, immunocytochemical studies were performed using receptor-specific antibodies. These studies demonstrated marked cell-surface expression of the TNF alpha-p55 receptor compared with expression of the TNF alpha-p75 receptor on human TSMC. Taken together, our results suggest that TNF alpha modulates agonist-induced calcium transients and induces human TSMC proliferation by specific activation of the TNF alpha-p55 receptor. Further studies addressing the cellular and molecular mechanisms regulating cytokine modulation of airway smooth muscle function may provide new insight into mechanisms that induce airway hyperresponsiveness in asthma.

PMID: 8679222 [PubMed - indexed for MEDLINE]

1: Biochem Biophys Res Commun. 1993 Jun 30;193(3):1191-7.

Agonist properties of a microbial superantigen peptide.

Pontzer CH, **Griggs ND**, **Johnson HM**.

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Staphylococcal enterotoxin A (SEA) binds to class II major histocompatibility complex (MHC) molecules and stimulates monocytes to produce tumor necrosis factor alpha (TNF alpha) and interleukin one (IL-1). We have examined the monocyte stimulatory activity of individual synthetic peptides encompassing the entire sequence of the SEA molecule. Only one peptide, SEA(121-149), induced both TNF alpha and IL-1 production at a concentration as low as 30 microM. Consistent with its effects on monocyte function, SEA(121-149) was shown to bind directly to class II MHC molecules on the surface of both monocytes and B cells, and its binding was inhibited specifically by native SEA. Further, polyclonal antibody to SEA(121-149) inhibited induction of TNF alpha by both SEA and toxic shock syndrome toxin one. Thus, we have identified SEA(121-149) as a peptide agonist of SEA monocyte stimulatory activity.

PMID: 8323541 [PubMed - indexed for MEDLINE]

1: Blood. 1996 Jul 15;88(2):690-6.

Tumor necrosis factor-alpha and FMLP receptors are functionally linked during FMLP-stimulated activation of adherent human neutrophils.

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Human peripheral blood neutrophils (PMN) plated onto fibrinogen and activated with FMLP release H2O2 and lactoferrin, a specific granule component, with parallel kinetics. Although tumor necrosis factor-alpha (TNF alpha) only primes PMN in suspension, it is a potent agonist of adherent PMN. Activation of adherent PMN by FMLP (10(-7) mol/L) stimulated detectable release of TNF alpha within 45 minutes of stimulation, with maximal release (45.5 pg/10(6) cells) detected by 90 minutes. TNF alpha release paralleled the release of both lactoferrin and H2O2. To determine if TNF alpha plays a role in H2O2 and lactoferrin release, we investigated the effect of anti-TNF alpha antibodies on FMLP-stimulated activation of adherent PMN. A neutralizing rabbit anti-TNF alpha antibody inhibited both H2O2 and lactoferrin release stimulated by FMLP, whereas rabbit IgG, anti-HLA-A,B,C, anti-CD 14, and anti-interleukin-8 antibodies were without effect. The simultaneous addition of TNF alpha (1,000 U/mL) with anti-TNF alpha antibody reversed the inhibition seen with anti-TNF alpha alone. Furthermore, treatment of PMN with either actinomycin D or cycloheximide resulted in partial (33%) inhibition of H2O2 and lactoferrin release, suggesting that protein synthesis is required for FMLP-mediated activation of adherent PMN. The addition of TNF alpha to either cycloheximide or of actinomycin D-treated PMN overcame the inhibition, indicating that the effect was specific for TNF alpha. The addition of antibodies against either the 55-or 75-kD TNF alpha receptors (referred to as p55 and p75, respectively) resulted in partial (32%) inhibition of FMLP-mediated activation of H2O2 and lactoferrin release, whereas a combination of both antibodies reduced their release to control levels. These data indicate that both p55 and p75 are involved in FMLP activation of adherent PMN. Taken together, these findings indicate that the production of TNF alpha and ligation of TNF alpha receptors are central to FMLP activation of PMN adherent to fibrinogen.

PMID: 8695817 [PubMed - indexed for MEDLINE]

1: Circ Res. 1996 Aug;79(2):286-94.

Thrombin potently stimulates cytokine production in human vascular smooth muscle cells but not in mononuclear phagocytes.

Kranzhöfer R, Clinton SK, Ishii K, Coughlin SR, Fenton JW 2nd, Libby P.

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Thrombosis frequently occurs during atherogenesis and in response to vascular injury. Accumulating evidence supports a role for inflammation in the same situation. The present study therefore sought links between thrombosis and inflammation by determining whether thrombin, which is present in active form at sites of thrombosis, can elicit inflammatory functions of human monocytes and vascular smooth muscle cells (SMCs), two major constituents of advanced atheroma. Human alpha-thrombin (EC50, approximately equal to 500 pmol/L) potently induced interleukin (IL)-6 release from SMCs. The tethered-ligand thrombin receptor appeared to mediate this effect. Furthermore, alpha-thrombin also rapidly increased levels of mRNA encoding IL-6 and monocyte chemotactic protein-1 (MCP-1) in SMCs. In contrast, only alpha-thrombin concentrations of > or = 100 nmol/L could stimulate release of IL-6 or tumor necrosis factor-alpha (TNF alpha) in peripheral blood monocytes or monocyte-derived macrophages. Lipid loading of macrophages did not augment thrombin responsiveness. Likewise, only alpha-thrombin concentrations of > or = 100 nmol/L increased levels of IL-6, IL-1 beta, MCP-1, or TNF alpha mRNA in monocytes. Differential responses of SMCs and monocytes to thrombin extended to early agonist-mediated increases in [Ca2+]i. SMCs and endothelial cells, but not monocytes, contained abundant mRNA encoding the thrombin receptor and displayed cell surface thrombin receptor expression detected with a novel monoclonal antibody. Thus, the level of thrombin receptors appeared to account for the differential thrombin susceptibility of SMCs and monocytes. These data suggest that SMCs may be more sensitive than monocytes/macrophages to thrombin activation in human atheroma. Cytokines produced by thrombin-activated SMCs may contribute to ongoing inflammation in atheroma complicated by thrombosis or subjected to angioplasty.

PMID: 8756006 [PubMed - indexed for MEDLINE]

1: Blood Cells Mol Dis. 1995;21(2):156-67.

Links

Thrombin enhances monocyte secretion of tumor necrosis factor and interleukin-1 beta by two distinct mechanisms.

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Thrombosis and disseminated intravascular coagulation (DIC) are common complications of infections. Abnormal activation of coagulation is due in part of expression of tissue factor on intravascular cells in response to cytokines, including interleukin-1 beta (IL1 beta) and tumor necrosis factor (TNF). Both TNF and IL1 beta are thought to play significant roles in producing the pathologic manifestations of sepsis. Therefore, we examined the effects of thrombin on TNF and IL1 beta secretion of monocytes, and the ability of monocyte products to promote tissue factor expression by endothelial cells. Human monocytes were treated with thrombin or a thrombin receptor agonist peptide (SFLLRN), and/or bacterial lipopolysaccharide (LPS). The agonists were removed, and monocytes cultured 18 hours. The monocyte-conditioned supernatants were assayed for TNF and IL1 beta antigen, and for their ability to induce tissue factor expression on human umbilical vein endothelial cells and the Ea.hy endothelial cell line. Thrombin alone did not promote monocyte TNF or IL-1 beta secretion. However, thrombin enhanced LPS-induced TNF and IL1 secretion. Supernatants from monocytes exposed to LPS plus thrombin promoted greater tissue factor expression on endothelial cells than supernatants from those treated with LPS only. SFLLRN did not increase TNF secretion in response to LPS, but did enhance LPS-induced IL1 beta secretion and tissue factor-inducing activity. Neither SFLLRN nor active thrombin augmented the level of mRNA for TNF above that induced by LPS alone. However, both increased the LPS-induced level of IL1 beta message. Thus, thrombin enhanced LPS-induced TNF and IL1 beta secretion by monocytes. Unexpectedly, the effects on these two cytokines were mediated by different mechanisms. Enhancement of LPS-induced IL1 beta secretion was largely mediated via the tethered ligand type thrombin receptor and correlated with an increase in the steady state level of mRNA. By contrast, enhanced TNF required proteolytically active thrombin, but was not mediated by the tethered ligand receptor. These data demonstrate that physiologically relevant amounts of thrombin can synergize with endotoxin to stimulate monokine release. Thrombin could thereby play a role in the complex network of mediators involved in the pathophysiology of sepsis. We speculate that limiting thrombin activity during DIC could be a beneficial adjunct in the management of sepsis.

PMID: 8846045 [PubMed - indexed for MEDLINE]

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A synthetic tumor necrosis factor-alpha agonist peptide enhances human polymorphonuclear leukocyte-mediated killing of Plasmodium falciparum in vitro and suppresses Plasmodium chabaudi infection in mice.

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A peptide corresponding to residues 70-80 of the TNF-alpha polypeptide was synthesized and shown to enhance human PMN-mediated killing of Plasmodium falciparum in vitro and reduced the Plasmodium chabaudi parasitemia in mice. Studies of the mechanism of action showed that the peptide, TNF(70-80), stimulated and primed PMN for an increased respiratory burst and release of granule constituents in response to a second agonist. The PMN-stimulatory activity of the peptide was inhibited by mAbs against the p55 and p75 TNF receptors and a TNF-neutralizing mAb. Analysis of PMN receptor expression showed that CR3 (CD18/CD11b) and Fc gamma RIII were upregulated by TNF (70-80), which was consistent with the peptide's ability to enhance parasite killing by PMN. The peptide, unlike TNF, did not increase the expression of adhesion molecules on endothelial cells and failed to promote binding of P. falciparum-infected erythrocytes to endothelial cells. TNF(70-80) also inhibited the TNF-induced increase in adhesion of P. falciparum-infected erythrocytes to endothelial cells. The results demonstrate that the host-protective effects of TNF can be retained while toxic effects are eliminated using a selected, characterized subunit of the cytokine.

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1: Cell Immunol. 1997 Mar 15;176(2):180-5.

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The immunomodulator AS-101 inhibits IL-10 release and augments TNF alpha and IL-1 alpha release by mouse and human mononuclear phagocytes.

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AS-101 is a tellurium-based compound with known immunomodulating properties. The ability of AS-101 to potentiate the effects of chemotherapeutic drugs and augment cytokine production in vivo has led to clinical trials on AS-101 which are currently being carried out in cancer patients. In the present study we show that AS-101 selectively augments the release of TNF alpha and IL-1 alpha and inhibits the release of IL-10 by lipopolysaccharide (LPS)-stimulated mouse peritoneal macrophages and human monocytes. It does not significantly affect the release of IL-6 or leukemia inhibitory factor (LIF). By itself AS-101 does not induce the release of any of these cytokines. Analysis of IL-10 and TNF alpha RNA levels using semiquantitative PCR reveals that AS-101 blocks the transcription of IL-10 mRNA, but does not significantly affect TNF alpha mRNA. Although both AS-101 and interferon (IFN)-gamma inhibit IL-10, AS-101, unlike IFN-gamma, does not prime macrophages for LPS-induced nitric oxide release and does not appear to significantly affect monocyte HLA-DR expression. Our data suggest that AS-101 is a partial IFN-gamma agonist and may explain the shift toward the release of Th-1 type cytokines observed in AS-101-treated patients.

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1: Infect Immun. 1993 May;61(5):2015-23.

Induction of early gene expression in murine macrophages by synthetic lipid A analogs with differing endotoxic potentials.

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Numerous lipid A analogs have been synthesized in an attempt to dissociate endotoxic activities from beneficial immunomodulatory activities. In the present study, we have evaluated select lipid A analogs in macrophages for their ability to induce a panel of lipopolysaccharide (LPS)-inducible genes to gain insights into the molecular mechanisms which underlie endotoxicity. We evaluated three monosaccharide lipid A analogs: SDZ MRL 953, an agonist with an improved therapeutic margin over endotoxin; SDZ 281.288, a more toxic analog; and SDZ 880.431, an analog with proven LPS-inhibitory activity. In addition, three disaccharide lipid A analogs (i.e., lipid IVA, SDZ 880.611, and SDZ 880.924) that differ in acylation and phosphorylation patterns were also examined and compared with synthetic lipid A. With the exception of SDZ 880.431, each of these structurally diverse analogs was able to induce the complete panel of LPS-inducible genes, specifically genes which encode tumor necrosis factor alpha (TNF-alpha), interleukin-1 beta, 75-kDa type 2 TNF receptor (D7), IP-10, D3, and D8. These results underscore that macrophage stimulation by lipid A analogs is permissive to considerable structural diversity. Structures with favorable therapeutic indices (SDZ MRL 953, SDZ 880.611, and SDZ 880.924) were not different from structures with poor therapeutic indices (lipid A, lipid IVA, and SDZ 281.288) with regard to gene induction. Nonetheless, the nontoxic SDZ MRL 953 was approximately 1,000-fold less potent than synthetic lipid A at inducing TNF-alpha secretion, and perhaps this contributes to the lack of toxicity exhibited by this compound. The ability of compound SDZ 880.431 to inhibit TNF-alpha secretion induced by both SDZ MRL 953 and smooth LPS suggests that the monosaccharide and smooth LPS share a receptor or a portion thereof. A pattern of protein tyrosine phosphorylation similar to that induced by LPS was stimulated by the monosaccharide SDZ MRL 953 and SDZ 281.288 and disaccharides lipid IVA, SDZ 880.924, and SDZ 880.611, providing evidence for a common signalling pathway.

PMID: 7683001 [PubMed - indexed for MEDLINE]

1: J Immunol. 1990 May 1;144(9):3513-7.

Selective activation of human monocytes by the platelet-activating factor analog 1-O-hexadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine.

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The capacity of platelet-activating factor (PAF) and its 2-O-methyl analog (methoxy-PAF) to activate human monocytes, neutrophils and platelets were compared. Both PAF and methoxy-PAF increased monocyte cytotoxicity toward WEHI 164 cells with a maximal increase in cell killing at 100 pM to 1 nM. Methoxy-PAF was slightly, but significantly, more potent than PAF for increasing cytotoxicity. PAF and methoxy-PAF increased monocyte release of TNF two- to three-fold above control release with no difference in their potency. Methoxy-PAF increased cell-associated TNF maximally after 2 to 3 h of incubation and increased TNF release maximally after 5 to 18 h of incubation. PAF induced release of the neutrophil granule enzyme beta-glucuronidase with maximal net release of 15 to 20% at 100 nM PAF whereas methoxy-PAF did not induce release of beta-glucuronidase. Similarly, 10 nM PAF induced 30% platelet aggregation whereas methoxy-PAF induced aggregation only at 1000-fold higher concentrations. Analysis of PAF and methoxy-PAF metabolism by monocyte and serum acylhydrolases indicates that methoxy-PAF is substantially more resistant than PAF to degradation by these enzymes. These observations indicate that methoxy-PAF activates monocytes selectively and suggest that this phospholipid or a related compound could be used for in vivo immunotherapy.

PMID: 2329280 [PubMed - indexed for MEDLINE]

1: J Interferon Cytokine Res. 1997 Oct;17(10):609-17.

Modulation of cytokine production by a selenoorganic compound (AE-22) in hyperreactive or hyporeactive bronchoalveolar leukocytes of asthmatics or lung cancer patients.

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We have found that many synthetic selenoorganic compounds, including ebselen, have immunotropic activity. These studies were designed to assess the effect of the analog of ebselen bis[2-pyridyl (2-carbamoyl) phenyl]diselenide (AE-22) on human leukocytes that may express various activation states. The cells were obtained from bronchoalveolar lavage (BAL) cells of patients with various inflammatory lung diseases. The AE-22-treated BAL cells from patients with bronchial asthma (n = 6) and with small cell lung cancer (SCLC) (n = 6) were compared with these in the peripheral blood leukocytes (PBL) from the same donors. The control group comprised 5 patients who underwent diagnostic examination and were free of any cancer or concomitant diseases. Secretion of TNF-alpha, IL-6, and IFN-gamma was considered as a marker of BAL or PBL cell activation. Different response of the cells and various effects of AE-22 were observed in relation to the origin and functional state of leukocytes. It was established that AE-22 can induce TNF-alpha, IL-6, and IFN-gamma in a dose-dependent manner in BAL cells and PBL isolated from healthy individuals. However, BAL cells were found to be less reactive than PBL as cytokine producers. In contrast, AE-22 had no effect on BAL cells obtained from patients with lung cancer, which were found to be hyporeactive to phytohemagglutinin and bacterial lipopolysaccharide and did not produce TNF-alpha, IL-6, or IFN spontaneously. The spontaneous release of cytokines by BAL cells from bronchial asthma patients, but not by PBL from the same individuals, was significantly ($p < 0.01$) higher than that from the cultures of healthy control subjects. The high secretion of cytokines by the locally activated BAL cells was significantly ($p < 0.01$) reduced after administration of AE-22. The results suggest that AE-22 has immunomodulatory activity. AE-22 can downregulate the hyporeactive BAL cells from asthmatics, but it appears to be inactive in BAL cells of cancer patients who can tolerate the cytokine inducers.

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1: J Biol Chem. 1997 May 16;272(20):12952-60.

Daunorubicin activates NFkappaB and induces kappaB-dependent gene expression in HL-60 promyelocytic and Jurkat T lymphoma cells.

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The anthracycline antibiotic, daunorubicin, can induce programmed cell death (apoptosis) in cells. Recent work suggests that this event is mediated by ceramide via enhanced ceramide synthase activity. Since the generation of ceramide has been directly linked with the activation of the transcription factor, NFkappaB, this was investigated as a novel target for the action of daunorubicin. Here we describe how treatment of HL-60 promyelocytes and Jurkat T lymphoma cells with daunorubicin results in the activation of the transcription factor NFkappaB. The effect of daunorubicin was evident following 1-2 h treatment, which was in contrast to the time course of activation obtained with the cytokine, tumor necrosis factor, where NFkappaB activation was detected within minutes of cellular stimulation. Activated complexes were shown to contain predominantly p50 and p65/RelA subunit components. Daunorubicin also induced IkappaB degradation and increased the expression of an NFkappaB-linked reporter gene. In addition, the drug was found to strongly potentiate the ability of tumor necrosis factor to induce an NFkappaB-linked reporter gene, suggesting a synergy between these two agents in this response. These events were sensitive to the iron chelator, deferoxamine mesylate (desferal), and the anti-oxidant and metal chelator pyrrolidine dithiocarbamate. A structurally related compound, mitoxantrone, which, unlike daunorubicin, is unable to undergo redox cycling in cells, also activated NFkappaB in a pyrrolidine dithiocarbamate-sensitive manner. A specific inhibitor of ceramide synthase, fumonisin B1, had no effect on daunorubicin induced NFkappaB activation at a range of concentrations previously reported to block apoptosis induced by this drug. However, this agent could inhibit increases in ceramide induced by daunorubicin, in addition to blocking ceramide synthase activity from HL-60 cells which was activated in response to daunorubicin treatment. These data therefore suggest that the effect of daunorubicin on NFkappaB is unlikely to involve ceramide, but may involve reactive oxygen species generated as a result of endogenous cellular processes rather than reductive metabolism of the drug. As NFkappaB may be involved in apoptosis, this effect may be an important aspect of the cellular responses to this agent.

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1: J Biol Chem. 1997 Jun 6;272(23):14914-20.

Activation of NF-kappaB by antineoplastic agents. Role of protein kinase C.

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Paclitaxel can induce tumor necrosis factor (TNF) and interleukin-1 gene expression, similar to lipopolysaccharides. Since lipopolysaccharide-induced expression of TNF is related to activation of NF-kappaB, we determined whether NF-kappaB could be activated by paclitaxel. In the human lung adenocarcinoma cell line A549, paclitaxel activated NF-kappaB in a dose-dependent manner with maximal activation after 2-4 h. Since paclitaxel could up-regulate TNF and interleukin-1 secretion and subsequent NF-kappaB activation could be caused by these cytokines, the effect of two other groups of anticancer drugs including vinca alkaloids (vinblastine and vincristine) and anthracyclines (daunomycin and doxorubicin), neither of which induce TNF or interleukin-1 gene expression, were examined. Like paclitaxel, vinblastine, vincristine, daunomycin, and doxorubicin each caused activation of NF-kappaB. Therefore, it is unlikely that activation of NF-kappaB caused by these agents or by paclitaxel is mediated via cytokine up-regulation. Furthermore, actinomycin D and cycloheximide, inhibitors of transcription and translation, respectively, did not inhibit paclitaxel-induced NF-kappaB activation. Several other transcription factors such as AP-1, AP-2, CREB, SP-1, or TFIID were not activated by antineoplastic agents demonstrating specificity of NF-kappaB activation. The involvement of both subunits in the NF-kappaB DNA binding complex was demonstrated by its abrogation by anti-p65 and by supershift by anti-p50 antibodies. Since protein phosphorylation is implicated in the activation of NF-kappaB, the effect of anticancer drugs on protein kinase C activity was measured. Vincristine, daunomycin, and paclitaxel significantly increased protein kinase C activity, and vinblastine and doxorubicin caused similar trends. Following treatment with antineoplastics (1-4 h), cytoplasmic IkappaBalpha degradation occurred concomitantly with translocation of p65 to the nucleus. Specific protein kinase C inhibitors (bisindolylmaleimide (GF109203X) and calphostin C) blocked the activation of NF-kappaB by each compound. Hence, protein kinase C activation may contribute to NF-kappaB activation by antineoplastic agents.

PMID: 9169462 [PubMed - indexed for MEDLINE]

1: Mol Pharmacol. 1998 Mar;53(3):402-7.

Ajoene, a compound of garlic, induces apoptosis in human promyeloleukemic cells, accompanied by generation of reactive oxygen species and activation of nuclear factor kappaB.

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The pharmacological role of garlic in prevention and treatment of cancer has received increasing attention, but thorough investigations into the molecular mechanisms of action of garlic compounds are rare. The present study demonstrates that ajoene, a major compound of garlic induces apoptosis in human leukemic cells, but not in peripheral mononuclear blood cells of healthy donors. The effect was dose and time dependent. Apoptosis was judged by three criteria, morphology of cells, quantification of subdiploid DNA content by flow cytometry, and detection of DNA fragmentation by gel electrophoresis. Ajoene increased the production of intracellular peroxide in a dose- and time-dependent fashion, which could be partially blocked by preincubation of the human leukemic cells with the antioxidant N-acetylcysteine. Interestingly, N-acetylcysteine-treated cells showed a 50% loss of ajoene-induced apoptosis. Moreover, ajoene was demonstrated to activate nuclear translocation of the transcription factor nuclear factor kappaB, an effect that was abrogated in N-acetylcysteine-loaded cells. These results suggested that ajoene might induce apoptosis in human leukemic cells via stimulation of peroxide production and activation of nuclear factor kappaB. This is a novel aspect in the biological profile of this garlic compound and an important step in elucidating the underlying molecular mechanisms of its antitumor action.

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CMLS Cellular and Molecular Life Sciences

Review

The therapeutic potential of tumor necrosis factor for autoimmune disease: a mechanistically based hypothesis

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Abstract. Excess levels of tumor necrosis factor-α (TNF-α) have been associated with certain autoimmune diseases. Under the rationale that elevated TNF-α levels are deleterious, several anti-TNF-α therapies are now available to block the action of TNF-α in patients with autoimmune diseases with a chronic inflammatory component to the destructive process. TNF-α antagonists have provided clinical benefit to many patients, but their use also is accompanied by new or aggravated forms of autoimmunity. Here we propose a mechanistically based hypothesis for the adverse events observed with TNF-α antagonists, and argue for the opposite therapeutic strategy: to boost or restore TNF-α activity as a treatment for some forms of autoimmunity. Activation defects in the transcription factor nuclear factor κB leave autoreactive T cells sensitive to TNF-α-induced apoptosis. Treatment with TNF-α, by destroying autoreactive T cells, appears to be a highly targeted strategy to interrupt the pathogenesis of type 1 diabetes, lupus and certain forms of autoimmunity.

Key words. NF-κB; TNF-α; apoptosis; autoimmunity.

Introduction and overview

Anti-tumor necrosis factor-α (anti-TNF-α) therapies have been introduced for treating moderate to severe rheumatoid arthritis, Crohn's disease and other chronic inflammatory disorders. The approved therapies (infliximab, adalimumab and entanercept) are monoclonal antibodies or inhibitory molecules that block TNF-α activity [1]. The therapeutic rationale behind their development is reduction of pro-inflammatory actions of the cytokine TNF-α, which is found elevated with other cytokines in autoimmune lesions [2–4]. But TNF-α is not just a pro-inflammatory cytokine. It has also been proposed to be an immunoregulatory molecule that can alter the balance of T regulatory cells [5]. What if normal or even elevated TNF-α activity plays an essential role as an immune regulator that diminishes or prevents autoimmunity? If that were the case, blocking the effects of TNF-α might turn out to be counterproductive in certain forms of autoimmunity or at select stages of the disease.

TNF-α's dual physiological roles, as pro-inflammatory and immunoregulatory, might explain why anti-TNF-α therapies present a complex picture: the therapies are effective for the majority of autoimmune patients with rheumatoid arthritis with end organ destruction due to inflammation, but they worsen or induce autoimmunity for a significant minority of these patients. That kind of adverse event is consistent with numerous animal and human studies showing that reducing TNF-α activity aggravates or initiates certain forms of autoimmunity.

Intrigued by the paradoxical findings, our laboratory and others sought to investigate TNF-α's underlying role in autoimmune pathogenesis. The mechanistic evidence

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suggests, contrary to prevailing wisdom, that boosting or restoring TNF-α activity – rather than blocking it – might be therapeutic for some forms of autoimmunity. In this review article, we present a mechanistically based hypothesis that TNF-α holds potential therapeutic value because of its demonstrated capacity in animal models of autoimmunity to selectively kill, by apoptosis, autoreactive (pathogenic) T cells but not normal cells. The selective vulnerability of autoreactive T cells to TNF-α-induced apoptosis appears to stem from a variety of errors in activating nuclear factor κB (NF-κB), which is a crucial transcription factor with anti-apoptotic effects (among many other immune functions). Because our findings and others show that autoreactive T cells continue to remain sensitive to TNF-α-induced apoptosis, treatment with TNF-α appears to be a highly targeted strategy to destroy autoreactive T cells and interrupt the pathogenesis of autoimmunity.

Anti-TNF therapies can exacerbate or induce new autoimmune disease

Evidence has accumulated from clinical trials that anti-TNF-α therapies can, under certain circumstances, promote rather than quell certain forms of autoimmunity. The evidence is strongest for multiple sclerosis (MS), with studies showing that anti-TNF-α therapies exacerbate its course. An early phase I safety trial of two patients revealed that one anti-TNF-α therapy transiently increased demyelinating lesions in the central nervous system (CNS) and immune activation in the cerebrospinal fluid (CSF) [6]. A double-blind, placebo-controlled phase II safety trial of 168 patients with MS found no benefit with another anti-TNF-α drug candidate and also found more frequent and earlier exacerbations. The annualized exacerbation rate was up to 50% greater in treated versus placebo patients [7]. A case of new-onset MS has been reported in a patient with juvenile rheumatoid arthritis treated with yet a third anti-TNF-α therapy [8]. MS and/or new onset demyelination disease are also adverse events with infliximab therapy in colitis and Crohn's disease [9, 10]. Infliximab therapy in 125 Crohn's patients results, after 24 months, in a high cumulative incidence (57%) of patients developing antinuclear antibodies, two patients developing drug-induced lupus and one patient developing autoimmune hemolytic anemia [11].

In rheumatoid arthritis, therapy with diverse therapeutic forms of TNF-α antagonists in all therapeutic forms is associated with relatively common and detectable autoimmune adverse events, including demyelinating disease, confirmed forms of MS, autoimmune hemolytic anemia, type 1 diabetes, a lupus-like syndrome, and cutaneous lupus rashes. Further, 11–57% of patients develop new or elevated antinuclear antibodies, usually shortly after therapy initiation or within 1 year [1, 2, 11–15]. Approximately 7–15% of patients develop new antibodies against double-stranded DNA [13, 15]. Case reports indicate onset of systemic lupus erythematosis or a similar syndrome at 6 weeks to 14 months after treatment initiation [14, 16–19]. Rheumatoid arthritis patients treated with anti-TNF-α therapies can also develop new onset autoimmune vasculitis [20, 21]. About 9–34% of patients with Crohn's disease in clinical trials of TNF-α antagonists develop antinuclear or double-stranded DNA antibodies [4, 22, 23]. Another trial of a TNF-α antagonist reported that 50% of treated Crohn's disease patients develop positive antinuclear antibodies [24]. Progression of lupus has been reported in several patients when TNF-α antagonists were not withdrawn [23]. Recently, a case of type I diabetes was reported in a 7-year old girl undergoing treatment of juvenile rheumatoid arthritis with a TNF-α antagonist [25]. The induction of new onset autoimmunity or the occasional worsening of autoimmunity is an apparent class effect of anti-TNF-α therapy and is not unique to any given TNF-α antagonist.

Low TNF activity may predispose to some forms of autoimmune disease

Several lines of investigation in humans and animals suggest that low TNF-α activity is associated with pathogenesis of some forms of autoimmunity. Low TNF-α activity might result from gene polymorphisms reducing TNF-α expression or disrupting its production. Low TNF-α activity also might result from excess production of soluble TNF receptors. Soluble TNF receptors bind to and inactivate TNF-α [26], effectively lowering TNF levels available to bind to membrane-bound receptors, which is a necessary step for activating intracellular signaling pathways.

Autoimmune researchers provided early insights about low TNF-α production being associated with disease activity in certain groups of patients and in spontaneous animal models of autoimmunity. Jacob and colleagues [27] searched for correlations between human major histocompatibility complex (MHC) haplotypes and predisposition to lupus. TNF-α genes are found within the MHC. Gene polymorphisms in the TNF region, with other genes in the region, appear to confer risk. Patients with certain MHC haplotypes show low levels of TNF production and an increased incidence of lupus nephritis. Patients with another MHC haplotype show high levels of TNF production and decreased incidence of lupus nephritis. The investigators did not know which genes in the MHC are responsible for the association.

These genetic findings in lupus patients are consistent with experimental findings in NZB mice, an animal model for lupus. A deficiency in TNF production, created

by backcrossing NZB mice with TNF-deficient mice, is associated with acceleration of disease [28]. Similar to human lupus, the TNF-α region of the MHC contains polymorphisms with risk for lupus expression [29]. Furthermore, the spontaneous rat model of diabetic and thyroid autoimmunity (BB rat) exhibits lowered expression of TNF [30]. The latter study also linked low TNF to defective T cell maturation; TNF-α is a critical cytokine in the normal T cell negative selection in the periphery but not the thymus [30, 31].

Higher levels of circulating TNF receptors are found in association with lupus activity [32, 33]. Serum levels of both types of TNF receptors (TNF-sR55 and TNF-sR75) are elevated and are better markers of disease activity than other laboratory or clinical parameters [32]. High serum levels of soluble TNF receptors effectively lower bioavailable TNF.

In MS patients, excess TNF receptor shedding has been found in the blood [34], suggesting lower bioavailability of TNF. Higher levels of soluble TNF receptors have also been found in sera and synovial fluids of patients with rheumatoid arthritis [35]. One study reported that because soluble TNF receptors were much higher in lupus than RA patients, the relative TNF-α deficiency might be predicted to be more severe with lupus [33].

Autoreactive T cells have heightened TNF-α-induced apoptosis linked to dysfuntional NF-κB regulation

The onset of autoimmunity may be driven in part by deficient presentation of self-peptides followed by escape from apoptosis of poorly educated, naïve T cells. The released cells eventually become activated and autoreactive upon encountering the peptides of the target organ. Evidence discussed below suggests that several autoimmune diseases have a common vulnerability to TNF-α exposure: their activated, autoreactive T cells are sensitive to TNF-α-induced apoptosis. The common vulnerability appears to be linked to various errors in NF-κB signaling. The errors, while having distinct origins depending on the underlying disease, render activated, autoreactive T cells sensitive to TNF-α-triggered apoptosis.

TNF-α – and interleukin-1 (IL-1) and lipopolysaccharide (LPS) can activate transcription factor NF-κB. Activated NF-κB, in turn, has been implicated in the regulation of genes contributing to cytokine generation, expression of cell surface adhesion epitopes, lymphocyte maturation, MHC class I antigen processing and presentation, and protection from apoptosis after exposure to TNF-α [36–40]. Recognizing that autoimmune diseases have cell-specific interruptions in NF-κB processing, depending on cell type and its state of activation (as discussed below), we propose a mechanistically based hypothesis about the etiology of many common features of autoimmunity, e.g., altered cytokine generation, delayed lymphocyte maturation and the overabundance of naïve T cells [36, 41–44], and interrupted MHC class I antigen processing and presentation defects [45].

In late-stage NOD mice with progression towards clinical disease, T cells have defects in activating NF-κB upon exposure to TNF-α. In normal T cells, activation of NF-κB occurs in the cytoplasm and requires intact proteasomes to cleave the active form of NF-κB from the inhibitory protein IkB-α (after phosphorylation and ubiquination). Once released, the active form of NF-κB is free to enter the nucleus and to express target genes that prevent cell death in response to TNF-α exposure. But NOD mice have defective proteasomes that are unable to liberate NF-κB from IkB-α [46–48]. Having defective proteasomes makes a subpopulation of T cells, after low-dose exposure to TNF-α, vulnerable to rapid death in culture or in vivo [48]. The possible therapeutic benefit of TNF-α therapy is well demonstrated in the NOD mouse model. With either direct TNF-α administration or TNF-α induction, murine autoimmune disease is reversed [49]. Conversely, in the same mouse model, blockade of TNF signaling accelerates autoimmune disease [50].

In type 1 diabetes patients, a new mutation has been found in a protein (SUMO4) that regulates NF-κB activity [51]. Guo and colleagues have shown that this newly identified protein variant alters IkB-α function in antigen-presenting cells and results in altered NF-κB regulation. Since proper proteasomal processing of IkB-α is similarly important for T cells with different forms of cell surface triggering, one can reasonably anticipate that the SUMO4 protein mutation will also result in altered NF-κB activity in the T cells of type 1 diabetic patients. In short, both murine and human diabetes appear to have defects in the processing of the same protein (IkB-α) and the same lymphoid cell-signaling pathway (NF-κB).

In human lupus, NF-κB is also altered. Subsets of T cells have decreased activation of NF-κB with stimulation [52]. In this disease, it is still unknown which steps along the NF-κB signaling pathway are altered.

Several other autoimmune diseases display gene polymorphisms or defects that might alter NF-κB activity. In Crohn's disease, mutations have been found in the NOD2 gene in antigen-presenting cells, and the mutations appear to confer susceptibility to disease onset [53, 54]. Intracellular NOD2 proteins are critical for NF-κB activation. Although it is not known at this time whether the NOD2 protein plays a role in normal or pathogenic T cells, errors in NOD2 function could lower NF-κB activity in T cells exposed to TNF-α, rendering them more vulnerable to TNF-α-induced apoptosis. Scleroderma, an autoimmune disease of the skin, has now been reported to have T cell subpopulations with altered NF-κB activity with associated accelerated apoptosis [55].

TNF protects against autoimmunity

A plethora of experimental studies show that TNF-α, directly or indirectly, protects against the onset of early autoimmunity in animal models of three autoimmune diseases. We define early autoimmune disease as immunologically active disease but prior to clinical onset. Systemic administration of TNF-α suppresses or prevents onset of spontaneous autoimmune disease in a murine model of lupus [56] and in the NOD model of type 1 diabetes [57–59]. TNF-α's therapeutic action in the NOD mouse also can be delivered locally [60]. TNF-α administered to type 1 diabetic-prone BB rats prior to disease appearance also protects from autoimmune progression [58]. TNF-α administration protects against the development of experimental autoimmune encephalomyelitis (EAE), a murine model of MS [61].

In further support of these findings, numerous experimental studies in the NOD mouse find that agents that induce TNF-α, such as bacillus Calmette-Guérin (BCG) or complete Freund's adjuvant (CFA), have the same or similar effect as direct administration of TNF-α: they protect against diabetes onset or against recurrent disease in the transplanted islet tissue [62–70]. The effect appears dose-related, as repeat dosing with BCG is more effective in preventing diabetes than a single dose [71]. Indeed, chronic TNF-α administration can quell or subdue active NOD autoimmunity, especially in late-stage disease or in settings of late disease where the pathogenic upstream pathogenic naïve cells have not been eliminated. TNF-α eliminates potent effector cells, but not all potentially pathogenic T cells in various stages of activation, including quiescent cells (naïve, autoreactive T cells). TNF-α induction with the immunomodulator AS101 also delays onset of lupus in an animal model [72].

Rationale for TNF as therapy in established autoimmune disease

The previous sections have presented evidence of anti-TNF-α strategies exacerbating some forms of autoimmunity and of pro-TNF-α strategies protecting against onset or exacerbation. The foremost questions are, What is TNF-α's mechanism of action in autoimmunity? Does the mechanism justify use of TNF-α as therapy and, if so, under what circumstances?

Our hypothesis is that TNF holds potential therapeutic value because of its capacity in animal models of autoimmunity to selectively kill autoreactive (pathogenic) T cells but not normal cells. Our laboratory was the first to show that NOD mice have a defect in regulation of transcription factor NF-κB. In those mice, NF-κB dysregulation makes the pathogenic T cells selectively vulnerable to TNF-α-induced apoptosis [46]. In that study we reported that cultured immune cells from NOD mice have a proteasome defect that traces to reduced or absent expression of LMP2, a protein that forms one of the proteasome's two catalytic subunits. A defective proteasome disrupts the NF-κB pathway in the cytoplasm. The pathway normally activates NF-κB, which protects cells from TNF-α-induced apoptosis (fig. 1). The proteasome defect, in contrast, leaves T cells incapable of activating NF-κB, which makes them vulnerable to TNF-α-induced apoptosis. Unlike B cells and other immune cells, T cells do not constitutively express the active form of NF-κB. Instead,

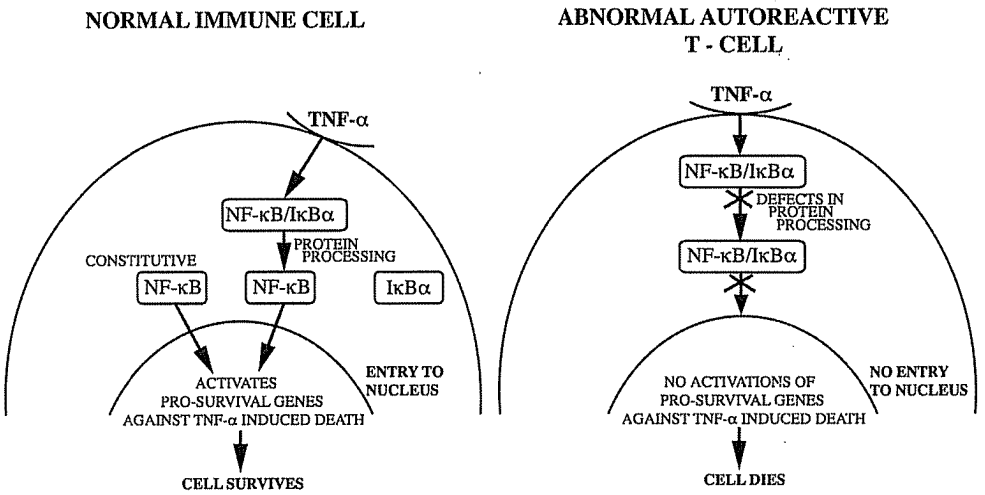

Figure 1. Response to TNF-α exposure by normal immune cells versus autoreactive (pathogenic) T cells. Normal cells either constitutively express the active form of NF-kB or have intact proteasomes that activate NF-kB by cleaving it from its bond with IkB-α. The active form of NF-κB then translocates to the nucleus to initiate expression of pro-survival genes, which counteract the apoptotic effects of TNF-α. In autoreactive (pathogenic) T cells, proteasomal defects prevent activation of NF-κB after TNF-α exposure. Without translocation of the active form of NF-κB into the nucleus, the cell dies because it is unable to express pro-survival genes.

they activate NF-κB upon exposure to TNF-α. Activation requires intact proteasomes to cleave the bond between IkB-α and NF-κB in order to produce the active NF-κB dimer (p65p50). Only the active form of NF-κB can translocate to the nucleus, bind to DNA and initiate expression of an array of anti-apoptotic proteins that counteract the pro-apoptotic effects of TNF-α [73]. If the active form of NF-κB fails to reach the nucleus, the T cell is unprotected from apoptosis when exposed to TNF-α. B cells and other immune cells are usually protected from TNF-α-induced apoptosis because they constitutively express the active form of NF-κB (fig. 1).

To confirm the importance of NF-κB in an animal model of diabetes, we found that freshly isolated NOD mouse T cells exhibit impaired degradation of IkB-α (via immunoblot analysis) and less binding of NF-κB to DNA (via a DNA binding assay) [46]. We also showed, via cell death assay, that a subset of splenocytes is highly sensitive to TNF-α-induced apoptosis in a dose-dependent manner. The greater the dose of TNF-α, the greater the degree of apoptosis (and hence less survival) in a select T cell subpopulation. Our finding of a dose-dependent, but cell-specific, increase in apoptosis with TNF-α, or a TNF-α-inducer, has been confirmed in vivo with NOD mice [74] and in vitro with cells from NZB mice [75]. Normal, freshly isolated splenocytes, on the other hand, are unaffected by TNF-α. Similarly, all populations of resting T cells, both in the NOD mouse and in normal mice, are resistant to TNF-α induction of death. Normal T cells are also resistant to TNF-α because they have intact proteasomes and normal production of NF-κB [46]. Also, nonactivated cells do not possess the intracellular signaling pathways leading to apoptosis after TNF-α exposure. B cells and other cells of the NOD mouse also survive after TNF-α exposure due to constitutive activation of NF-κB by pathways other than the T cell-specific proteasomal activation pathway [76, 77].

Abnormal NF-κB activity has been found in humans with lupus and Crohn's disease [52–54]. Only NOD mice appear to have the proteasome defect as the origin of abnormal NF-κB activity, but the role of the altered the NF-κB pathway and specific IkB-α regulation appears similar to that found in humans with type 1 diabetes [46, 51, 78, 79]. For instance, the proteosomal defect in the NOD animal model is due to a promoter mutation in the MHC-linked proteasome subunit LMP2 [79]. This proteasome defect, conferring altered NF-κB induction in mature T cells, is developmentally specific because it appears gradually and after only 5–6 weeks of age [47]. The appearance of the defect is consistent with the time course of autoimmunity. NOD mice do not manifest autoimmunity until at least 5–6 weeks of age, when they develop insulitis, and by 8 weeks of age, when they produce autoantibodies. The LMP2 subunit proteasome defect in NOD mice is apparently found in multiple cell types of lymphoid origin [80], but its impact in the proteasome (namely, dysregulation of NF-κB and TNF-α-induced cell death) is required for T cells, as contrasted with macrophages and B cells [76, 81, 82]. The lineage specificity of NF-κB activation via IκBα degradation is what makes TNF-α selectively lethal to one type of cell and, more specifically, to one subpopulation of activated T cells.

Selective TNF-induced apoptosis of pathogenic cells in the NOD mouse

The pathogenesis of type 1 diabetes stems in part from impaired activation of transcription factor NF-κB in a subpopulation of activated T cells. This defect makes these cells susceptible to rapid TNF-α-induced death. Repeat TNF-α dosing is necessary for disease suppression when the autoimmune disease is in late stages and the upstream naïve pathogeneic cells can still form the endstage highly activated autoreactive CD8+ T cells. These cells are generated from a subset of naïve T cells that are resistant to TNF-α until they become activated. Working under the hypothesis that autoreactive but naïve T cells in the NOD mouse would survive after TNF administration or induction, we tested a two-pronged strategy to determine whether permanent disease elimination was possible by eliminating both naïve and activated autoreactive T cells.

We tested this two-pronged treatment strategy in endstage diabetic NOD mice: (i) a single administration of TNF-α, or CFA (an inducer of TNF-α) to eliminate activated autoreactive T cells, and (ii) treatment with matched complexes of MHC class I molecules and self-peptides to kill newly emerging naïve, autoreactive T cells [49, 83]. The strategy permanently reversed (i.e., cured) diabetes by returning the animals to normoglycemia [49]. Remarkably, stable and robust pancreatic islet cells reappeared in the pancreas as a result of de novo regeneration from endogenous or exogenously administered adult stem cells [84]. The effectiveness of a similar, two-pronged treatment strategy in a diabetes animal model also has been found by von Herrath and colleagues [85].

Mechanisms of Treatment Efficacy

We report here further in vivo and in vitro analyses in the NOD mouse to confirm the mechanisms by which treatment was successful, especially in regard to killing pathogenic memory (i.e., previously activated) T cells. We examined the immunological pathways underlying successful treatment, characterized treatment variables, and evaluated the role of distinct peripheral lymphoid T cell subpopulations in disease and confirmed their selective death with therapy.

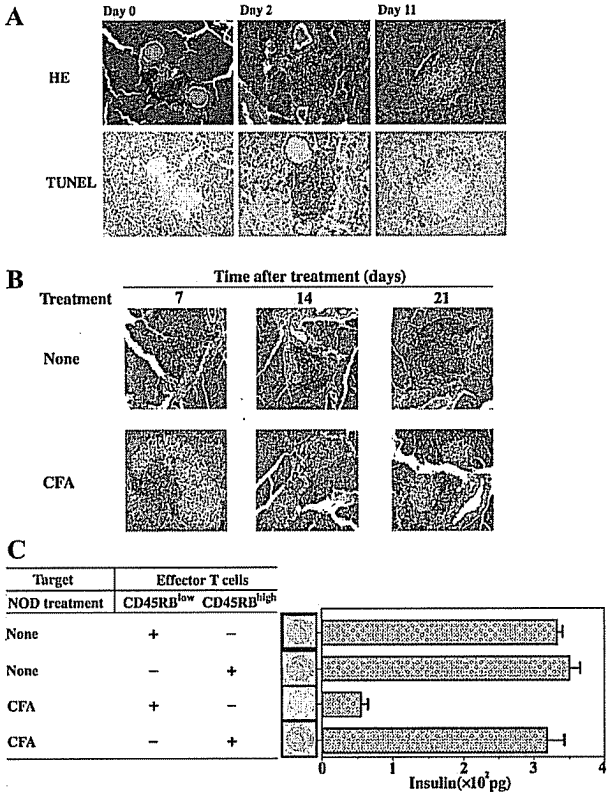

Figure 2. Islet-directed autoreactivity in vitro of T cell subpopulations from diabetic NOD mice subjected to various treatments and effect of CFA treatment on invasive insulitis in vivo. (*A*) Hematoxylin-eosin (HE) and TUNEL staining of paired serial sections derived from the pancreases of pre-diabetic NOD female mice treated (or not, day 0) with a single injection of CFA and sacrificed 2 or 11 days later. (*B*) HE staining of pancreatic sections derived from the pancreases of late pre-diabetic NOD female mice treated (or not, day 0) with a single injection of CFA and sacrificed 7, 14 or 21 days later. (*C*) Cytotoxicity assays were performed with dispersed islet cells from young NOD females. CD45RB^{low} and CD45RB^{high} subpopulations of effector T cells were isolated from the splenocytes of untreated diabetic NOD female given a single injection of CFA. Islet-directed autoreactivity was assessed by ELISA measurement of insulin release and by an associated colorimetric change of the culture medium. Data are means ± SD of triplicates from an experiment that was repeated a total of five times using different donor animals. The bottom row represents islets without any added T cells

We first found, as expected, that treatment with the TNF-α-inducer CFA rapidly produced massive apoptosis of the T cells responsible for destructive insulitis of diabetic mice (fig. 2A). The treatment also diminished insulitis by day 11. The animals were NOD females at 15–20 weeks of age, when pancreatic autoreactivity (but not hyperglycemia) is evident. We examined tissues of the pancreas 2 or 11 days after treatment with CFA alone. Paired serial tissue sections were stained by hematoxylin-eosin (HE) to detect lymphoid infiltrates and by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) technique to detect apoptotic cells. Untreated NOD animals (day 0) show destruction of islets by extensive and invasive lymphoid infiltrates. TUNEL staining (day 0) showed that the invasive infiltrates consists of viable lymphocytes. However, 2 days after CFA injection, the invasive lymphoid infiltrates are still evident, but they are now composed of apoptotic cells. By day 11, the invasive insulitis is eliminated from the central region of islets, and islet structures become evident. TUNEL staining again revealed that the islet core is viable and that the invading lymphocytes are dead. Thus, CFA treatment induces striking apoptosis of lymphoid cell infiltrates–without harming the underlying pancreatic islet structure. But CFA alone is incapable of curing established diabetes. Follow-up times in excess of 20 days after CFA injection revealed the slow and gradual reappearance of invasive insulitis (data not shown), presumably due to proliferation and invasion of naïve autoreactive T cells exposed to islet antigen.

We also used direct HE staining of pancreatic tissue to determine the extent of lymphoid infiltrates over a longer period of time (fig. 2B). Untreated NOD mice develop progressively greater insulitis over the course of 21 days. But CFA treatment eliminates invasive insulitis as early as 7 days after therapy initiation. The elimination of pathogenic insulitis persists, even with a single dose therapy, through day 21.

Two populations of pathogenic T cells, in different stages of activation, are killed by different means

In the next set of studies, we examined the impact of treatment on two subpopulations of autoreactive T cells in the mouse: CD45RB^{low} (memory or activated T cells) and CD45RB^{high} (naïve T cells). Specifically, we wanted to prove that TNF-α or TNF-α inducers only kill memory T cells (CD45RB^{low}), but have no effect on naïve autoreactive T cells.

High expression of the surface marker CD45RB identifies cells unexposed to antigen, whereas low expression signifies past or ongoing exposure to antigen [86, 87]. The maintenance of CD45RB is tightly controlled by proper and continuous MHC class I and self-peptide expression in the periphery [88]. These later memory T cells with additional surface marker identification are sometimes referred to as T regulatory cells and are recognized as deficient in autoimmunity. Human type 1 diabetes is characterized by an abundance of naïve T cells or deficiency of T regulatory T cells [41–44]. Again, this abnormality, as with the NOD mouse, is due to defective MHC class I and self-peptide presentation for central and continuous peripheral T cell selection [45, 49, 89].

While our treatment selectively eliminates distinct subpopulations, the evidence described here focuses on destruction of only one subpopulation of autoreactive T cells, namely, memory T cells, when they are isolated from treated NOD mice and examined by in vitro as-

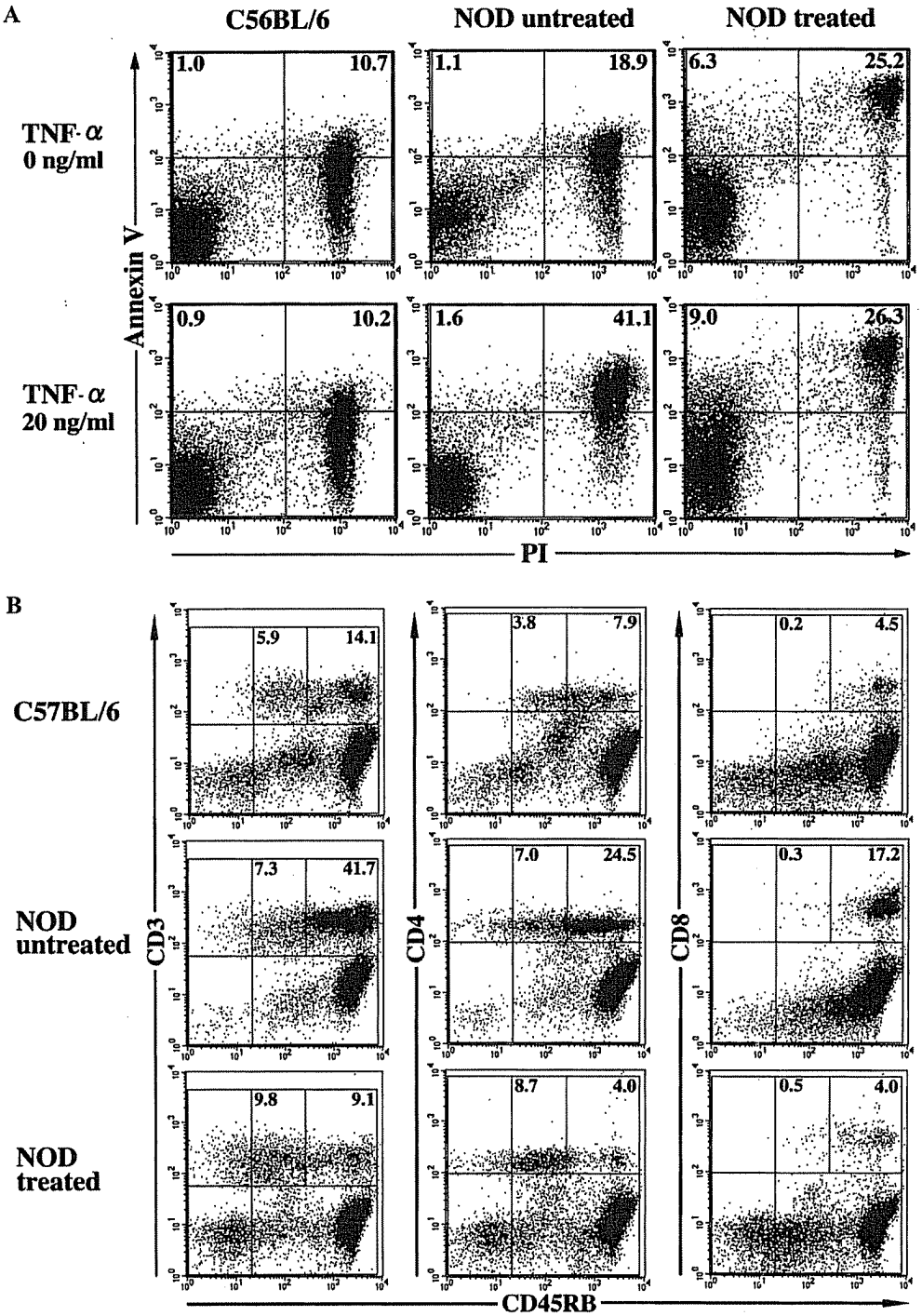

Figure 3. Elimination of autoreactive T cells and normalization of naïve and memory T cell subpopulations in NOD mice with long-term disease reversal with CFA and MHC-peptide complexes. (*A*) Effect of TNF-α on the survival of spleen cells derived from a control C57BL/6 mouse, an untreated 20-week-old NOD female or a NOD female with long-term disease reversal. Cells were incubated in the absence or presence of TNF-α (20 ng/ml) for 24 h before staining with annexin V and propidium iodide (PI) followed by flow cytometric analysis. Similar results were obtained with a total of eight mice with long-term disease reversal. (*B*) Flow cytometric analysis of $CD3^+CD45RB^+$, $CD4^+CD45RB^+$ or $CD8^+CD45RB^+$ splenocytes isolated from a C57BL/6J mouse, an untreated NOD female with diabetes or a treated NOD female with long-term restoration of normoglycemia. Similar results were obtained with a total of four mice with long-term disease reversal.

says (fig. 2C). We isolated each T cell subpopulation (CD45RB^{low} and CD45RB^{high} cells) from animals after two treatment conditions: no treatment or CFA-only treatment. Using an islet cytotoxicity assay, we assessed the ability of each subpopulation to lyse dispersed islets from a young NOD pre-diabetic mouse. A T cell subpopulation that is eliminated by our treatment would have less capacity to kill dispersed islets, which, in turn, would release less insulin. Islet death is indicated by the degree of released insulin in the media, as measured by enzyme-linked immunosorbant assay (ELISA).

With no treatment, CD45RB^{low} (memory T cells) and CD45RB^{high} (naïve autoreactive T cells) are equally capable of lysing islets. With CFA-only treatment, CD45RB^{low} (memory T cells) lyse fewer islets. This implies that CD45RB^{low} cells must have been previously killed by CFA treatment–presumably from the defect in the activation of endogenous NF-κB. In contrast, the CFA-only treatment does not kill the CD45RB^{high} cells, because their capacity to lyse islets is as vigorous as that in untreated animals. We conclude that CFA selectively kills only memory autoreactive T cells – a finding consistent with other reports of selective vulnerability of memory T cells to TNF-α-induced apoptosis [90, 91]. Our findings are also supported by those of Qin and colleagues [74], who found that BCG, another TNF-α inducer, selectively kills CD4+ and CD8+CD45RB^{low} cells in NOD mice. Naïve pathogenic cells are killed by MHC class I and self-peptide re-exposures (data not shown).

We performed another assay of apoptosis – by flow cytometry after staining with propidium iodide and fluoroscein isothiocyanate (FITC)-conjugated annexin V – to confirm that (i) treatment successfully eliminates cells that are selectively sensitive to TNF-α and (ii) no treatment still leaves cells from diabetic mice sensitive to TNF-α-induced apoptosis. We isolated splenocytes from a treated NOD mouse, an untreated NOD mouse and a normal C56BL/6 mouse to compare their in vitro sensitivity to TNF-α incubation. As shown previously [46, 48], incubation of normal (C57BL/6) spleen cells with TNF-α has no effect on cell viability. The proportion of apoptotic cells is 10.2% in the presence of TNF-α and 10.7% in its absence (fig. 3A). In contrast, there is a dramatic rise in apoptosis with TNF-α incubation, from 18.9 to 41.1%, in splenoctyes from an untreated non-diabetic NOD female. There is no increase in apoptosis in splenocytes from a previously CFA-treated female NOD mouse who had become normoglycemic for >180 days. The apoptosis levels remain nearly the same in the absence or presence of TNF-α. This implies that earlier CFA treatment had effectively killed one subpopulation of TNF-α-sensitive cells. The fact that the magnitude of apoptosis is similar, yet similarly high (25.2% and 26.3% with or without TNF-α, respectively), requires further explanation. We reported a similar finding in our earlier study ([49], Fig. 7a). We believe there is a separate subpopulation of splenocytes, apart from autoreactive T cells, which undergoes spontaneous death when cultured. Several studies of peripheral blood in lupus or systemic sclerosis patients also uncovered apoptotic lymphocytes or monocytes upon placement in culture [92–98]. The findings suggest that other immune cell populations besides autoreactive T cells are altered in autoimmunity and, consequently, that other strategies may been needed – above and beyond TNF-α therapy.

Both diabetic NOD mice and humans with diabetes previously have been shown to possess an overabundance of unstimulated or naïve T cells [41, 43, 44], defined in mice by the surface phenotype CD3+CD45RB^{high}. We therefore evaluated the impact of treatment with CFA and MHC-peptide complexes on the cell surface phenotype of peripheral CD45RB-expressing T cells. Our expectation was that treatment would reduce the overabundance of naïve cells. Using flow cytometric analysis of splenocytes, we first showed that a normal mouse (age- and sex-matched) displays about a 1:2 ratio of the two phenotypes: 5.9% are CD45RB^{low} (memory T cells) and 14.1% are CD45RB^{high} (naïve T cells). An untreated diabetic mouse, however, displays a 1:6 ratio, with 7.2% CD45RB^{low} and 41.7% of CD3+ cells CD45RB.^{high} This confirms that untreated diabetes is associated with a striking overabundance of naïve T cells (fig. 3B). The overabundance of naïve T cells is also apparent on analysis of the CD4+ and CD8+ subpopulations from the same mouse. But 180 days after treatment with CFA and MHC class I-peptide complexes, the normal ratio of low to high for CD3+ CD4+ or CD8+ T cells is resumed almost to normal in an NOD female restored to normoglycemia (fig. 3B). In other words, brief treatment, assists in bringing down the percentage of naïve cells, even after long-term follow-up.

TNF eliminates a highly pathogenic T cell subpopulation

Finally, we performed several adoptive transfer experiments to test whether treatment, in vitro or in vivo, successfully eliminates pathogenic lymphocytes. First, we established the validity of our model of adoptive transfer. We transferred splenocytes (2×10^7), which had been isolated and pooled from diabetic NOD females, to each of 21 irradiated young (4- to 8-weeks old) male pre-diabetic NOD recipients. All 21 recipients developed severe hyperglycemia within 10–25 days, with a mean ± SD of 18.4 ± 3.6 days (fig. 4, panel A), a finding commonly reported with this experimental model. Likewise, the male recipients also demonstrated pancreatic islet pathology featuring extensive and pronounced insulitis (fig. 4, panel C). The pathology is indistinguishable from that in NOD females after spontaneous onset of disease at 20–30-weeks of age.

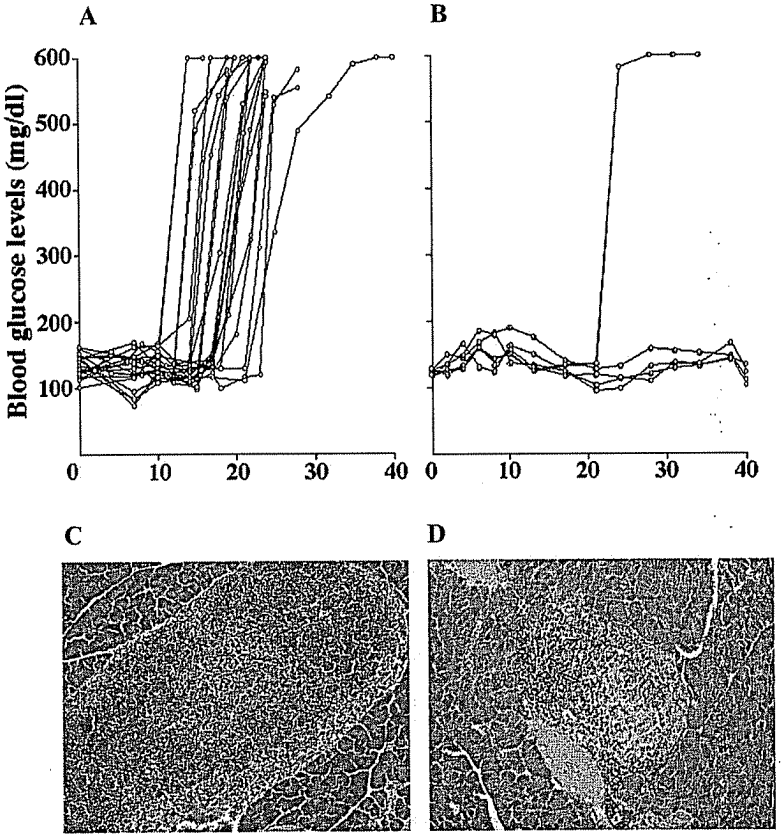

Figure 4. Elimination of autoreactive T cells by TNF-α treatment in vitro as demonstrated by adoptive transfer. Splenocytes isolated from diabetic NOD females were left untreated (panels *A* and *B*) or incubated for 24 h with TNF-α (20 ng/ml) in vitro (panels *B* and *D*) before adoptive transfer into young NOD males. The isolated cells were then transferred to young NOD males. The effects of adoptive transfer on blood glucose concentration (panels *A–B*) and on pancreatic islet histology (panels *C–D*) were determined.

Then we tested the impact of treating cells in vitro with TNF-α prior to adoptive transfer. We found that treatment nearly eliminated pathogenic cells. Splenocytes from diabetic NOD females, which had been treated with TNF-α for 24 h before adoptive transfer, failed to produce hyperglycemia in four (80%) of the five male recipients for at least 40 days (fig. 4, panel B). This finding is consistent with TNF-α-induced apoptosis of disease-causing cells. Examination of pancreatic histology 40 days after cell transfer revealed mild or moderate invasive insulitis (fig. 4, panel D). Thus, while culture with TNF-α before adoptive transfer appears to have eliminated most of the highly pathogenic cells, they were not completely eliminated since the infusion also contained naïve pathogenic T cells.

In summary, the findings presented here support the existence of autoreactive T cells, in particular memory autoreactive T cells ($CD45RB^{low}$), which are susceptible in vitro or in vivo to TNF-α-induced apoptosis. Disease reversal by combination treatment (CFA plus MHC class I-peptide complexes) assists in the normalization of peripheral ratios of naïve versus memory T cells in all populations of T cells (CD4 or CD8). Several distinct assays were used to confirm the death of autoreactive T cells with therapy, the preservation of normal pancreatic tissue and the restoration of normoglycemia. The findings support the therapeutic use of TNF or TNF inducers, such as BCG and CFA, to kill autoreactive T cells in diabetes and possibly other autoimmune diseases.

Model of TNF in autoimmune disease and therapy

In normal people, autoreactive T cells are destroyed in the bone or thymus during the process of negative selection. The process of apoptosis occurs through the T cell receptor, fas ligand or other death pathways [74, 99]. While TNF is constitutively express in the thymus [100], it does not appear to play an essential role in negative selection of early precursor T cells [31].

In people with autoimmune disease, autoreactive T cells survive after having evaded negative selection. In the NOD mouse model, their escape from negative selection occurs because of a proteasomal defect that alters protein processing, including processing of self-antigens for display on the cell surface of autoreactive T cells. Without appropriate display of self-antigens, autoreactive T cells

avoid being seen as 'self', and thus avoid targeted death. Their survival leads to onset or worsening of autoimmunity. But the underlying proteasomal defect in these cells also renders them exquisitely sensitive to TNF-α-induced apoptosis. Because of the dysfunctional proteasome, autoreactive T cells, of the activated type, do not form NF-κB, the transcription factor that would ordinarily protect them from TNF-α-induced apoptosis. Lacking functional NF-κB, they remain sensitive to TNF-α-induced apoptosis. It is that continued sensitivity to TNF-α-induced apoptosis that can be exploited for therapeutic purposes. We propose therapy with TNF-α, or TNF-α inducers, to selectively kill autoreactive T cells in type 1 diabetes, lupus and MS. Treatment with TNF-α appears to offer a highly targeted strategy to destroy autoreactive T cells and interrupt the pathogenesis of autoimmunity. TNF does not appear to harm normal T cells or other tissues, presumably due to their active form of NF-κB. The level and timing of TNF-α therapy, as well as disease status, appear to be key variables in therapy. Pathogenic memory T cells are especially sensitive to TNF-α-induced apoptosis. Their destruction required only a single dose of therapy with a TNF-α-inducer with a short half-life. That should allay concerns that TNF-α levels become so high that they might induce inflammation.

Our findings show the benefits of TNF-α for autoimmune diabetes, but they also show that TNF-α alone is insufficient to cure the condition with one single dose. TNF-α does not eliminate the naïve autoreactive T cells, which will become pathogenic on exposure to self-antigens, because they are yet sensitive to TNF-α. To prevent eventual self-tissue recognition and activation, naïve T cells must be killed by an additional therapy with a separate death pathway.

The evidence from lupus and certain other autoimmune diseases suggests that low TNF-α levels worsen or bring on disease. The reason may be that the deficiency of TNF-α allows continued survival of autoreactive T cells. The cells previously might have escaped negative selection because of improper processing of self-antigen. In these diseases, introducing or inducing TNF-α may succeed as a therapeutic strategy.

Anti-TNF-α strategies are successful for a large percentage of rheumatoid arthritis patients. Often these patients are in advanced forms of disease, and their painful symptomatology may be driven by a strong inflammatory component to the disease process. While the mechanism of action is not fully understood, it could be that prior to treatment, TNF-α levels are so high in patients' lesions that reducing pro-inflammatory effects of TNF-α provides clinical benefit. Inflammation may be the major source of the discomfort and deformity at this stage of disease.

Our hypothesis holds that despite the apparent benefits of reducing inflammation, anti-TNF-α therapies would have no effect on the underlying autoimmune disease or might actually worsen it. Indeed, the adverse event reporting discussed previously documents the common occurrence of autoantibodies to self-antigens and the occurrence of new autoimmune disease with therapy, usually in the form of lupus, MS or even diabetes. Autoreactive T cells, according to our model, are expected to survive and proliferate in a low TNF-α environment. An alternative explanation for the success of anti-TNF-α therapies in certain autoimmune diseases such as rheumatoid arthritis is that this disease may have underlying defects that are distinct from and unrelated to the NF-κB signaling defects found in type 1 diabetes, lupus and MS.

Another alternative explanation comes from recent data suggesting that some forms of anti-TNF-α drugs bind a form of TNF-α that sits on the exterior surface of the T cell, a transmembrane form of TNF-α itself. This newly identified form of TNF-α on the external cell surface membrane allows direct anti-TNF-α binding, and this binding directly kills the T cell [101–104]. This form of T cell killing is not believed to be specific only to autoreactive T cells and most likely represents an effect similar to an immunsuppressive drug. At this early time the T cell death pathway for this killing is undefined.

These mechanistic findings about anti-TNF-α therapies appear to underscore our hypothesis that a new generation of therapies should strive for selective apoptosis of activated, autoreactive T cells.

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Research paper

Methods to characterize lymphoid apoptosis in a murine model of autoreactivity

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Abstract

The immune system is shaped by the random generation of lymphocytes followed by apoptosis of self-reactive cells, a process termed negative selection. The survival of these pathogenic cells in the periphery can elicit autoreactivity. We describe the development of a biomarker assay for the detection of pathogenic subpopulations of lymphoid cells in adult non-obese diabetic (NOD) mice based on disease-specific alterations in spontaneous or triggered cell death. Utilizing improved methods of cell separations, two distinct lymphoid cell subpopulations with increased susceptibility to apoptosis were identified and quantified. A subpopulation of $CD8^+$ T cells that constitutes ~3–7% of the total $CD8^+$ T cell population underwent apoptosis on exposure to low concentrations of TNF-α. Such cells were exclusively detected only in NOD mice with histologic signs of active autoreactivity. The non-T cell compartment of NOD immune system, although resistant to TNF-α-induced apoptosis, contained a subpopulation of B cells with spontaneous death by culture alone. The refined detection of small numbers of lymphoid cell subsets with quantifiable differences in apoptosis provides a possible immune biomarker for monitoring disease activity or treatment interventions.

Keywords: Apoptosis; Cytokines; Splenocytes; Biomarkers; TNF-α

1. Introduction

Apoptosis plays a central role in the development and peripheral shaping of the immune system. During lymphocyte development, autoreactive cells are eliminated by apoptosis, a process termed negative selection. Regulation of apoptosis has been found to be abnormal in a diversity of human and murine autoimmune diseases, allowing the survival of autoreactive cells in the periphery (Ohashi, 2003). A failure of antigen presenting cells to properly display a full repertoire of self-peptide in the MHC classes I or II antigen presenting structures allows self-reactive T cell formation. The cellular responses to certain death promoting cytokines that shape T cell selection in the periphery, such as free TNF-α (non-receptor bound), are deficient in certain forms of human and murine autoimmunity and may also allow self-reactive cells to survive and cause dis-

Abbreviations: NOD, non-obese diabetic; MHC, major histocompatibility complex; FBS, fetal bovine serum; PI, propidium iodide; C57, C57BL/6; FSC, forward scatter of cells; SSC, side scatter of cells.

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ease (Gabay et al., 1997; Jurewicz et al., 1999). The induction of TNF-α appears to be a highly targeted strategy to destroy autoreactive T cells in vivo, as has been shown in two different spontaneous murine models of autoimmunity (Jacob and McDevitt, 1988; Ryu et al., 2001). The therapeutic administration of anti-TNF-α in humans worsens certain forms of autoimmunity or induces new forms of autoimmunity in some clinical settings (van Oosten et al., 1996; Anonymous, 1999; Sandborn and Hanauer, 1999; Sicotte and Voskuhl, 2001; Shakoor et al., 2002; Anonymous, 2003).

The routine detection of freshly isolated autoreactive cells in humans or mice with autoimmunity has been difficult. In large part, antigen-specific T cells have been sought as indicators of active disease. These cells are extremely rare, require a prior knowledge of specific antigen specificity, and in vivo cell numbers vary depending upon the stage of disease and the type of autoreactivity. As an alternative to the detection of antigen-specific T cells, the detection of broader populations of poorly selected lymphoid cells with a phenotype of altered apoptosis offers an alternative. Indeed for many years, human and murine researchers observed altered apoptosis in unseparated blood or spleen cells populations in a diversity of autoreactive diseases (Emlen et al., 1994; Rose et al., 1997; Perniok et al., 1998). To date, these observations have not been expanded into more tightly controlled biomarker assays that allow an individual animal or human to be evaluated for the degree of the defect or the cellular subpopulation possessing the altered apoptosis defects. The ability to detect autoreactive immune cells directly would make it possible to monitor these cells during disease progression and treatment.

The development of new cell-based autoimmune detection methods based on induced or accelerated apoptosis requires freshly isolated lymphoid cells. These fresh cells need to have reproducible viability and yield prior to the assay start. Lymphoid cells in murine models of autoimmunity have traditionally been harvested from the spleen. A growing literature shows the inadequacy of traditional splenocyte isolations (Hsueh et al., 2002). This highlights the need to develop methods that eliminate harsh gradient cell separations, lengthy centrifugations, and applications of toxic red blood cell lysis methods or cell transfers that create time delays prior to analysis. The recent successful multi-center efforts to meticulously refine the most basic steps of mouse splenic B lymphocyte cell separations demonstrate the success of standardized fresh splenocyte studies (Sambrano et al., 2002). These concerted efforts on devising new lymphocyte isolations methods have yielded viable cells at the start, reproducible cellular representations and allowed multi-center efforts to define normal signal transduction networks of B-lymphocytes from the spleens of normal mice (Hsueh et al., 2002). A similar standardization of all lymphoid cell isolations is necessary for the development of reliable apoptotic markers for autoimmune subpopulations.

The present study develops a new method for the rapid isolation of splenocytes with high yield and consistent viability from diabetes-prone NOD and control mice. We then optimized culture conditions for these fresh cells in order to quantify two forms of lymphoid cell death i.e. cell death induced by TNF-α and spontaneous apoptosis in vitro. With this approach, we have identified a quantifiable subpopulation of T cells, with co-expression of CD8, that selectively undergo cell death on exposure to TNF-α. We also identify a subpopulation of non-T cells that selectively undergo cell death with culture alone. The procedures and culture conditions described are conveniently transferable and provide a suitable beginning for the format of similar quantitative studies of the numbers of autoimmune cells with altered apoptotic programs.

2. Materials and methods

2.1. Animals

Female NOD mice were obtained from Taconic Farms (Germantown, NY), and C57BL/6J (C57) and BALB/c mice were from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed with NOD mice older than 12 weeks but before the onset of hyperglycemia, which typically occurs at 20 to 30 weeks of age. The NOD mice were maintained under pathogen-free conditions and screened for the onset of diabetes by monitoring of body weight and blood glucose concentration. Diabetes was diagnosed when two consecutive blood glucose concentrations exceeded 300 mg/dl.

2.2. Isolation and culture of splenocytes

Splenocyte isolation was optimized to yield the highest splenocyte viability and maximum overall cell yield. Mice were killed by cervical dislocation, and the spleen was removed through an abdominal incision, placed in a sterile petri dish containing RPMI medium supplemented with 10% FBS, and gently inflated by repeated injection of 1 to 2 ml of the medium with a 22-gauge needle. The fluid that leaked out of the spleen,

which contained dislodged splenocytes, was collected. Additional splenocytes were collected by gentle extrusion of tissue with blunt-tip forceps through a small opening created at one end of the spleen; the loose clumps of tissue were readily dissociated into single cells by repeated gentle pipetting. The collected cells were then passed through a 40-μm mesh filter to yield a crude splenocyte preparation. Purified splenocytes or lymphocyte subpopulations were cultured at 34 °C and 95% humidity in RPMI medium supplemented with 10% FBS and antibiotics.

2.3. Removal of RBCs

RBCs were removed from the crude splenocyte preparation in one of three ways: (i) The cells were resuspended in 10 ml of NH_4Cl lysis buffer [140 mM NH_4Cl, 17 mM Tris–HCl (pH 7.65)], incubated for 10 min at room temperature, and then washed once with RPMI medium containing 10% FBS. (ii) Cells suspended in RPMI medium containing 10% FBS (5 ml) were layered on top of 5 ml of Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) in a 15-ml conical centrifuge tube and centrifuged at 1300×*g* for 10 min at 4 °C, after which those present at the interface of the two layers were collected and washed once with 10 ml of RPMI medium containing 10% FBS. (iii) Cells (5×10^7) suspended in RPMI medium containing 5% FBS and were incubated for 15 min at 4 °C with magnetic beads conjugated with rat mAbs to mouse Ter119 (Miltenyi Biotec, Auburn, CA), washed once, and applied to a magnetic LS column (Miltenyi Biotec); the cells that passed through the column were washed once with RPMI medium supplemented with 10% FBS.

2.4. Fractionation of splenocyte subpopulations

For the separation of T cells from other splenocytes, we used a Pan T Cell Isolation Kit (Miltenyi Biotec), which includes a mixture of magnetic beads linked to antibodies specific for CD45R (B cells), DX5 (NK cells), CD11b (dendritic cells, monocytes–macrophages, granulocytes), and Ter119 (erythrocytes) and therefore removes virtually all non-T cells from a mixed cell population. For a similar negative selection of non-T cells, we used a mixture of magnetic beads conjugated with antibodies to CD90 (T cells) and to Ter119 (erythrocytes). For the positive selection of B cells or monocytes–macrophages, we used magnetic beads conjugated with antibodies to CD19 or to CD11b, respectively (Miltenyi Biotec). CD4 and CD8 T cells were isolated by positive selection using magnetic beads conjugated with antibodies against CD4 or CD8, respectively (Miltenyi Biotec).

2.5. Quantitation of cell viability, cell loss and apoptosis

Cell preparations were analyzed with a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) after culture for 20–24 h in RPMI medium supplemented with 10% FBS or in RPMI medium with or without 50 ng/ml mouse TNF-α added (Sigma). Apoptotic cells were detected by staining either with propidium iodide (PI) alone or with a mixture of PI and FITC-conjugated annexin V (TACS Annexin V-FITC Apoptosis Detection Kit; R&D Systems, Minneapolis, MN). In the latter approach, early apoptotic cells were defined as cells positive for staining with annexin V only, whereas late apoptotic cells were defined as cells positive for staining with both PI and annexin V. Cell viability was also assessed by staining with trypan blue; portions of cell suspension (25 μl) were diluted fivefold with 0.05% trypan blue (Sigma, St. Louis, MO) in PBS and examined with a hemacytometer. All samples were also studied for total cells remaining in case the dead apoptotic cells were no longer part of the PI positive cell population.

2.6. Statistical analysis

Comparisons between groups were performed with the one-tailed Student's *t*-test. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Increased death rate of NOD splenocytes in culture

Freshly isolated unstimulated splenocytes die in culture (Sun et al., 1992; Perandones et al., 1993; Zhang et al., 1995; Rinner et al., 1996; Sodja et al., 1998). With this fact as a starting point for the detection of subpopulations of autoreactive cells with an increased susceptibility to apoptosis, we isolated cells from the spleen of C57 and late-stage prediabetic NOD mice (12 to 17 weeks of age). RBCs were initially removed by density gradient centrifugation with Ficoll-Paque Plus. The purified splenocytes were cultured at a density of 5×10^6 cells per well (100 μl) in flat-bottom 96-well plates and their viability was evaluated at various times by staining with trypan blue. Unstimulated control (C57) and NOD splenocytes both gradually died in

culture. However, the rate of viability loss was markedly greater for NOD splenocytes than for the C57 cells (Fig. 1).

Given that the viability of primary splenocytes depends on cell density, we repeated the cell survival assays at different cell densities. In this instance, we removed RBCs from the crude splenocyte preparations with the use of magnetic beads conjugated with antibodies to Ter119, a procedure that is less harsh as compared to both Ficoll gradient centrifugation and NH_4Cl-induced RBC lysis (see below). The splenocytes were plated at densities of 10,000, 25,000, 50,000, or 100,000 cells per well (100 μl) in U-bottom 96-well plates and cultured for 24 h, after which cell viability was determined by staining of dead cells with PI and flow cytometry (see below). The viability of both C57 and NOD splenocytes increased with cell density (Fig. 2A). The viability of the NOD splenocytes, however, was again markedly lower than that of the C57 cells at all cell densities with the exception of 10,000 cells per well. Taking into account these data as well as the economic utilization of cells, we chose a cell density of 25,000 cells per well in U-bottom 96-well plates as our standard condition for characterization of cell death. The U-shaped bottom of the culture wells causes the cells to settle in the middle of the wells, resulting in a high local density even at low cell numbers (Fig. 2B); in contrast, the cells are situated farther apart in flat-bottom wells at the same seeding density.

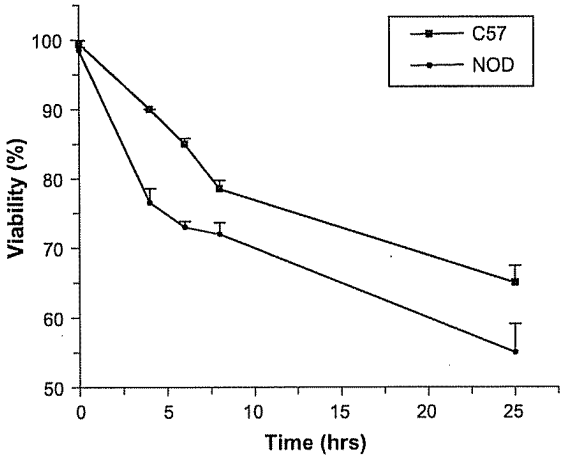

Fig. 1. Comparison of the rates of loss of viability for NOD and C57 splenocytes in culture. Crude splenocyte preparations were freed of RBCs by density gradient centrifugation on Ficoll-Paque Plus and then cultured at a density of 5×10^6 cells per well (100 μl) in flat-bottom 96-well plates. Cell viability on the basis of trypan blue exclusion was determined at the indicated times. Two representative experiments are shown performed in triplicates. A total of seven separate experiments were performed. The (●) indicate values for NOD mice; the (■) indicate values for C57 mice.

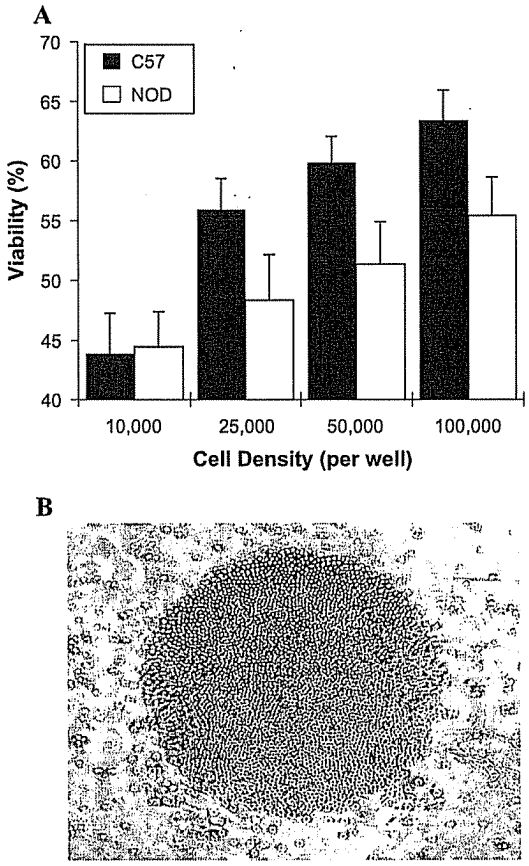

Fig. 2. Cell density dependence of NOD and C57 splenocyte viability in culture. (A) RBCs were removed from crude splenocyte preparations with magnetic beads linked to antibodies to Ter119, and the remaining cells were seeded at densities of 10,000 to 100,000 cells per well (100 μl) in U-bottom 96-well plates. Cell viability was determined after 20–24 h by staining of dead cells with PI and flow cytometry. Data are from three experiments done on separate days. (B) NOD splenocytes plated at a density of 10,000 cells per well as in (A) were examined by phase-contrast microscopy after culture for 24 h. The U-shape of the well bottom causes the cells to settle at the middle of the well, creating a high local cell density. Original magnification, 100×.

3.2. Dependence of splenocyte viability on the method of RBC removal

Given that an overall low viability of freshly isolated splenocytes might be expected to hamper the ability to detect and quantify subpopulations of cells with an increased susceptibility to apoptosis, we sought to optimize the method for cell isolation from the spleen of normal mice. For the dissociation of splenic tissue, we first tried the traditional method of processing the

spleen through a cell strainer with a 40-μm mesh. However, the viability of cells isolated by this procedure, as measured by trypan blue exclusion, was variable and frequently as low as 85%. We therefore evaluated several variations of this standard method to try to improve the initial cell viability. We eventually selected a procedure that involves inflating the spleen with culture medium to loosen the tissue, followed by harvesting of the tissue by scraping and further dissociation of cell clumps by gentle pipetting (see Materials and methods). This approach consistently yielded a high initial viability of >95% as determined by various assays.

We next evaluated various methods for removal of RBCs from the crude splenocyte preparation. Cell death was monitored on the basis of flow cytometric analysis of cells stained with annexin V and PI. We thus compared the viabilities of splenocytes that had been cultured for 20–24 h after the removal of RBCs by NH_4Cl-induced lysis, Ficoll gradient centrifugation, or magnetic separation with antibodies to Ter119 (which is expressed on mature mouse RBCs and erythroid precursor cells, but not on lymphoid or myeloid cells). In the experiment shown in Fig. 3A, the viability of the cultured splenocytes was lowest for the cells purified by NH_4Cl lysis (34.4%), intermediate for the cells purified by density gradient centrifugation (51.2%), and highest for the cells purified by magnetic cell sorting (72.5%). Statistical data from repeat experiments ($n=21$, 9 and 14 independent experiments) show the NH_4Cl separations, with respect to viability, were inferior to Ficoll ($p=0.005$) and Ficoll separation viability was inferior to magnetically separated cells ($p=0.00006$). NH_4Cl was also inferior to magnetically separated cells ($p=5\times10^{-9}$). We selected magnetic cell sorting with antibodies to Ter119 as

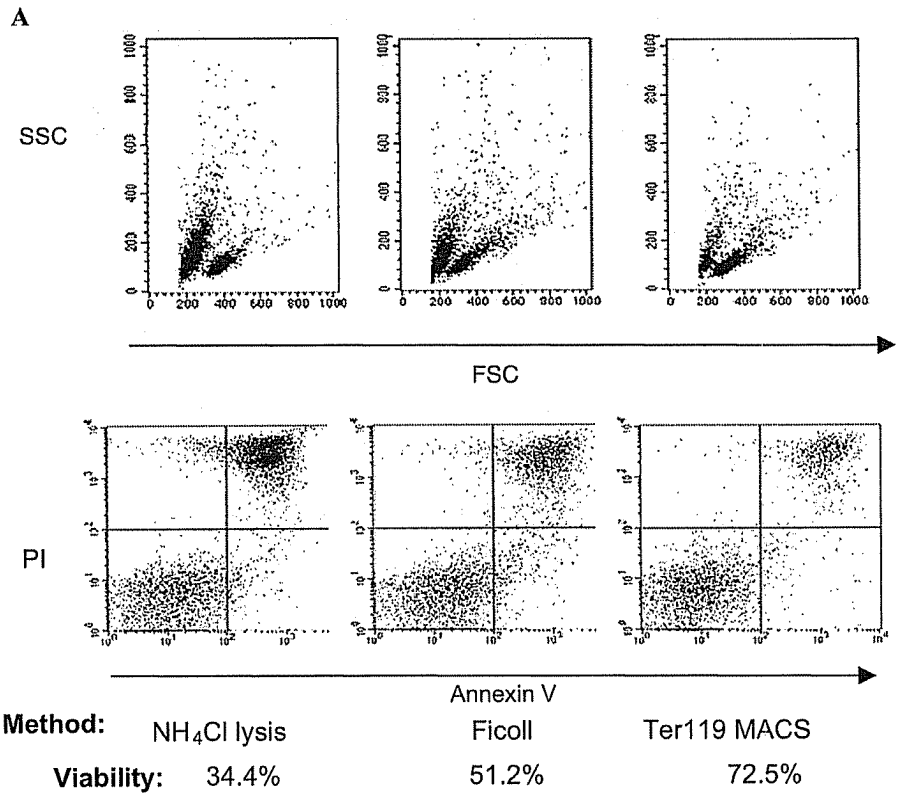

Fig. 3. Dependence of splenocyte viability on the method of RBC removal and localization of live and dead splenocyte populations on flow cytometric plots A. RBCs were removed from crude normal splenocyte preparations by either NH_4Cl-induced lysis, Ficoll gradient centrifugation, or magnetic separation (MACS) with antibodies to Ter119. The purified splenocytes were then cultured for 24 h before determination of cell viability by flow cytometric analysis of cells stained with annexin V and PI (live cells were defined as cells negative for both PI and annexin V staining). Results from a representative experiment are shown in (A). FSC, forward scatter; SSC, side scatter. (B). Localization of live and dead splenocyte populations on flow cytometric plots of SSC versus FSC. Purified C57 splenocytes were cultured for 20–24 h, stained with annexin V and PI, and analyzed by flow cytometry. Plots of SSC versus FSC (i) as well as of PI versus annexin V staining for ungated cells (ii) or for cells gated on the basis of the R1 (iii) or R3 (iv) regions identified in the scatter plot are shown.

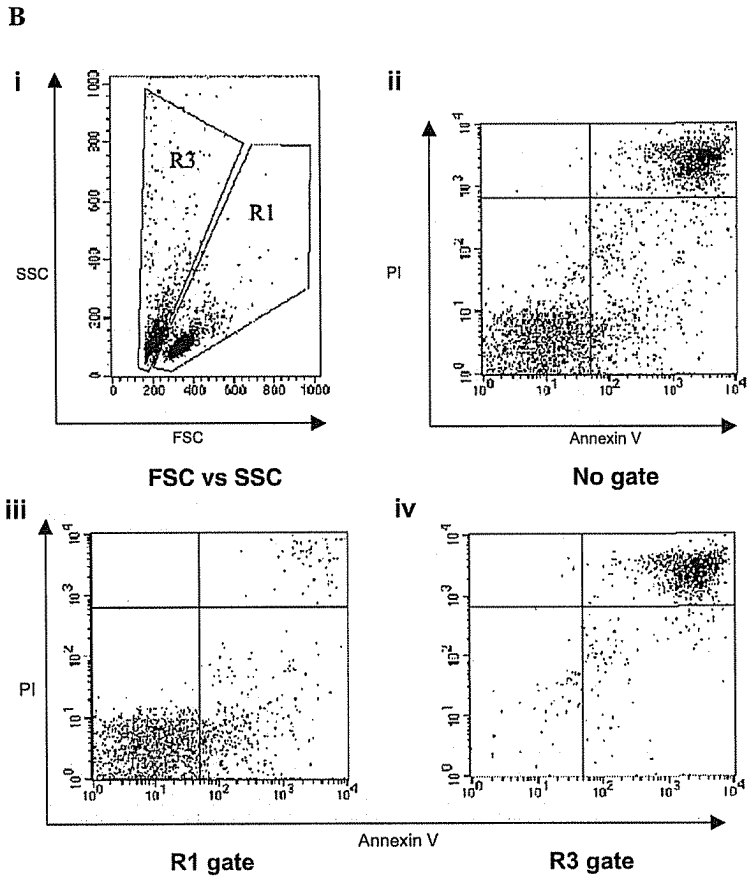

Fig. 3 (*continued*).

our approach for removal of RBCs from crude splenocyte preparations.

We determined the locations of live and dead populations of normal splenocytes (purified by magnetic cell sorting and cultured for 20–24 h) on flow cytometric plots of forward scatter (FSC) versus side scatter (SSC). The cells appeared as two major populations (R1 and R3) on such plots (Fig. 3B, i). The plot of PI versus annexin V staining for ungated cells revealed populations that remained unstained (live) or that stained with both annexin V and PI (late apoptotic or dead) (Fig. 3B, ii). We then determined the plots of PI versus annexin V staining for the cells corresponding to regions R1 (Fig. 3B, iii) and R3 (Fig. 3B, iv). These plots revealed that most live cells localized to the R1 region, whereas most late apoptotic and dead cells were restricted to the R3 region. This observation is consistent with the fact that cells shrink and become irregular in shape during apoptosis, resulting in a decrease in FSC and an increase in SSC. Thus, by gating for R1 or R3, it was possible to identify predominantly live or predominantly dead cell populations, respectively.

3.3. Differential apoptotic susceptibility of splenic T cells and non-T cells

The spleen contains a variety of immune cell types, which can be classified as T cells (mostly $CD4^+$ and $CD8^+$ cells) or non-T cells (mostly B cells and monocytes/macrophages). To determine whether the increased death rate of NOD splenocytes in culture was generalized or restricted to specific cell subsets, we used magnetic cell sorting to obtain T cell and non-T cell fractions of splenocytes from NOD and control mice. Both cell fractions were isolated by negative selection. For the isolation of T cells, we thus removed B cells, NK cells, dendritic cells, monocytes–macrophages, granulocytes, and erythroid cells. For the isolation of non-T-cells, we removed T cells and erythroid cells. The use of negative selection ensured that the cells to be studied had not been exposed to antibodies that bind to surface receptors and thereby trigger cell activation.

We first compared the viabilities of a mixture of T cells and non-T cells (using only anti-Ter119); of T cells

alone; and of non-T cells alone, that had been isolated from splenocytes of control mice and cultured for 20–24 h. In the experiment shown in Fig. 4A, the viabilities determined by flow cytometric analysis of PI- and annexin V-stained cells were 69.0% for T cells, 37.7% for non-T cells, and 51.9% for the mixture of T cells and non-T cells. The rate of spontaneous death in culture was thus greater for non-T cells than for T cells. We also determined the viability of T cell and non-T cell subpopulations of control and NOD splenocytes after culture for 20–24 h at different seeding densities (Fig. 4B). The viability of T cells was relatively independent of cell density and did not differ substantially between control mice and NOD mice although NOD T cells overall had a slightly higher viability at all densities. In contrast, the viability of the non-T cells increased with cell density and was greater for the control cells than for the NOD cells. This latter observation was found to be statistically significant on repetition of the experiment (Fig. 4C), with the viabilities of control and NOD non-T cells averaging $53.7 \pm 3.0\%$ and $47.9 \pm 3.6\%$, respectively.

To determine which non-T cell types were most sensitive to death in culture, we further fractionated the non-T cell population into cells that express either CD11b (monocytes–macrophages) or CD19 (B cells)

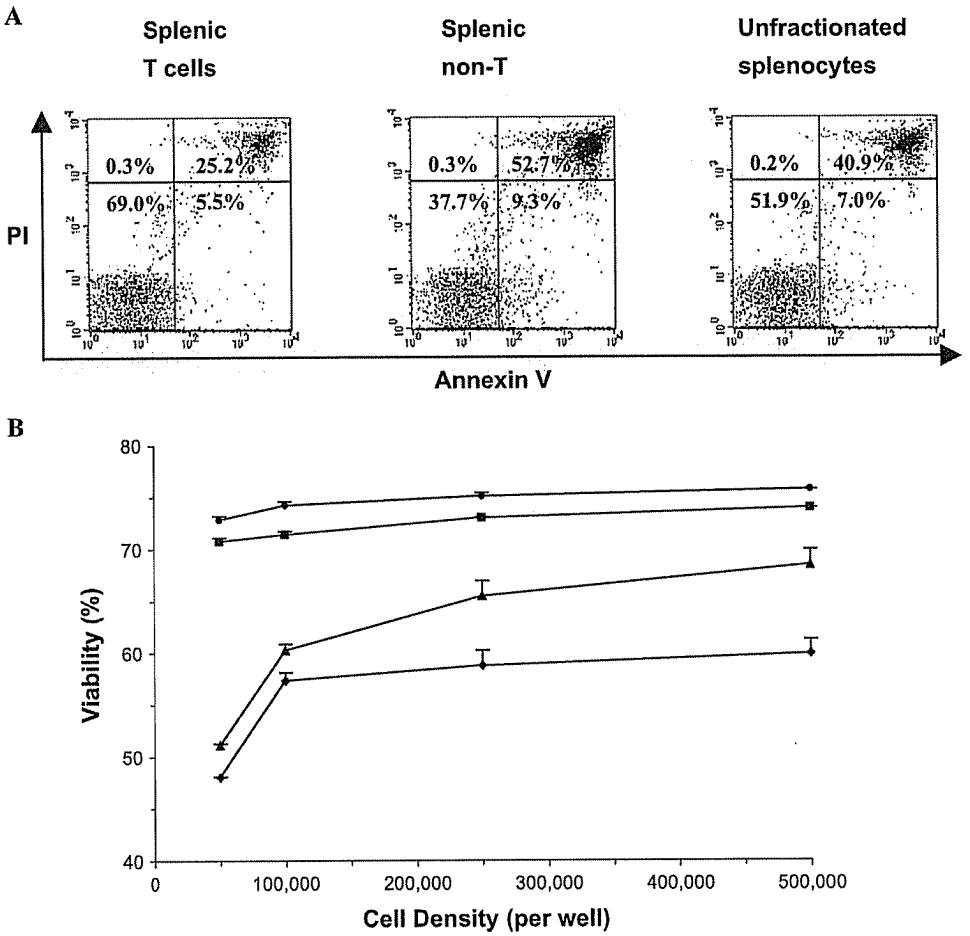

Fig. 4. Comparison of the susceptibilities of T cells and non-T cells isolated from C57 (squares, triangles) and NOD (circles, triangles) splenocytes to spontaneous apoptosis in culture. (A) Freshly isolated splenocytes of C57 mice were fractionated into T cells (circles, squares) or non-T cells (triangles, diamonds), or simply freed of RBCs (nonfractionated splenocytes), and the various cell subpopulations were then cultured for 20–24 h before staining with PI and annexin V and analysis by flow cytometry. (B) T cell and non-T cell fractions isolated from C57 or NOD splenocytes were plated at the indicated densities and cultured for 24 h, after which cell viability was determined as in (A). Data are from 3 representative experiments. (C) Viabilities of non-T cell fractions of C57 and NOD splenocytes determined after culture for 20–24 h as in (A). Data are means ± S.E.M. of values from 12 independent experiments. (D) The non-T cell subpopulations of C57 and NOD splenocytes were further fractionated into monocytes or B cells, and the resulting cell fractions were cultured and assayed for cell viability as in (A). Data are from 3 representative experiments and are expressed as the percentage of initially viable cells that died during culture.

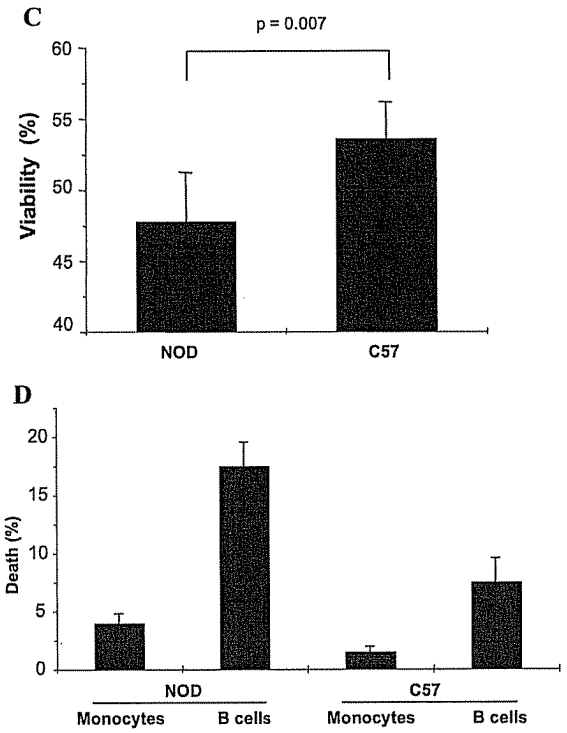

Fig. 4 (*continued*).

by positive selection. Whereas culture of each cell subpopulation for 24 h resulted in substantial cell death, the extent of such death was greater for B cells than for monocytes–macrophages for both NOD and control cells. Greatest cell death was observed for B cells from NOD (Fig. 4D).

3.4. Enhanced sensitivity of a subset of NOD splenocytes to TNF-α-induced apoptosis

Flow cytometric analysis of cells stained with a combination of PI and annexin V is able to distinguish late apoptotic or dead cells (positive for both PI and annexin V) from live cells (negative for both PI and annexin V) and early apoptotic cells (PI negative, annexin V positive). However, given that: (1) this approach cannot distinguish between late apoptotic and dead cells; (2) that annexin V binds nonspecifically to B cells (Dillon et al., 2000); (3) that we did not detect necrotic cells (PI positive, annexin V negative) in our death assays; and (4) that FITC–annexin V is relatively expensive for large-scale use, we adopted a simpler flow cytometric analysis of cell death that is based on FSC of PI-stained cells (see below).

We previously showed by both trypan blue exclusion and flow cytometric analysis of PI- and annexin V-stained cells that NOD splenocytes isolated by Ficoll gradient centrifugation after NH_4Cl treatment contain a subpopulation of cells that exhibit an increased sensitivity to TNF-α-induced apoptosis (Hayashi and Faustman, 1999; Hayashi et al., 2000). To confirm this defect with our newly developed cell isolation and death detection protocols, we removed RBCs from crude control (C57) or NOD splenocyte preparations by magnetic cell sorting with anti-Ter119 and then plated the purified splenocytes in U-bottom 96-well plates at a density of 25,000 cells per well in 100 μl of RPMI medium in the absence or presence of mouse TNF-α (50 ng/ml). After culture for 24 h, the cells were stained with PI (2 μg/ml in PBS) and analyzed by flow cytometry. Plots of PI fluorescence versus FSC revealed two spatially distinct populations of live and dead cells (regions R1 and R2, respectively; see Fig. 5). Cellular debris (low FSC values) was detected to the left of region R1 but was ignored in our analysis (Fig. 5A). We counted the number of events in regions R1 and R2 and used the results to calculate percentage viability. Whereas culture with TNF-α increased the viability of C57 splenocytes for 50.6% to 54.1% (a change of 6.9%), it reduced that of NOD splenocytes from 50.2% to 47.3% (a change of −5.8%) (Fig. 5B). A protective effect of TNF-α on normal mouse splenocytes especially CD8 cells has been described previously (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996; Kuhtreiber et al., 2003).

3.5. NOD T cells are sensitive to TNF-α induced apoptosis; a defect in the CD8 subpopulation

Using the PI-FSC viability assay, we next determined whether the T cell or non-T cell subpopulation is responsible for the increased sensitivity of unseparated NOD splenocytes to TNF-α-induced apoptosis. We thus isolated T cells and non-T cells from splenocytes of late prediabetic NOD mice and normal C57 mice by negative selection, cultured them for 24 h in the absence or presence of TNF-α (50 ng/ml), and then determined their viability. Whereas C57 splenic T cells showed a minimal or no change in viability in response to TNF-α, the NOD splenic T cells showed a statistically significant decrease in viability (Fig. 6A, B). In contrast, there was no significant difference in TNF-α sensitivity between the non-T cell subpopulations of C57 and NOD splenocytes (Fig. 6C).

We further fractionated the T cell population into $CD4^+$ or $CD8^+$ cells by positive selection with antibo-

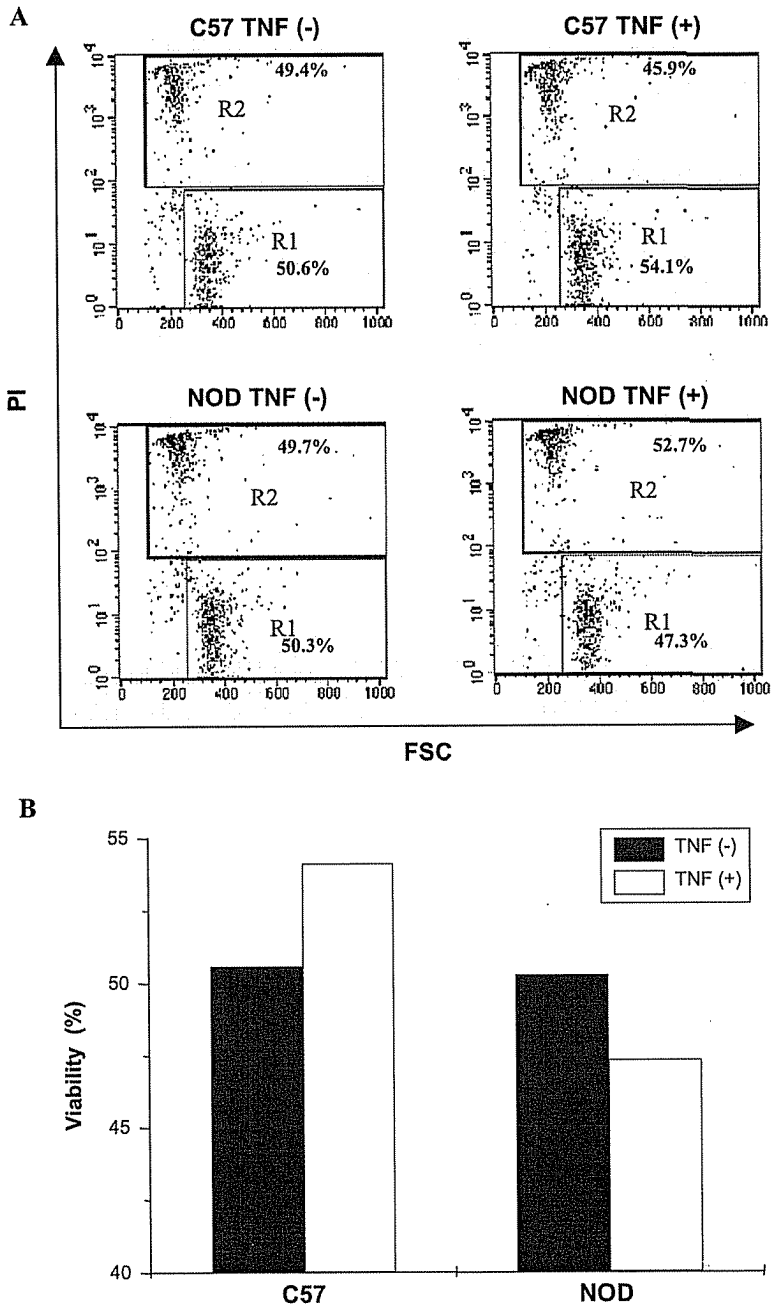

Fig. 5. Increased sensitivity of a subpopulation of NOD splenocytes to TNF-α-induced apoptosis in culture. (A) Freshly isolated splenocytes from C57 and NOD mice were cultured for 20–24 h at an initial density of 25,000 cells per well in U-bottom 96-well plates containing RPMI medium with or without TNF-α (50 ng/ml). The cells were then stained with PI and analyzed by flow cytometry. Plots of PI fluorescence versus FSC resolved the splenocytes into two spatially distinct populations of live (R1) and dead (R2) cells. (B) Percentage viability of C57 and NOD splenocytes for the experiment shown in (A). Data are from a representative experiment. Statistics are presented in Fig. 6.

dies to these antigens and magnetic cell sorting. The purified cells were cultured for 20–24 h with or without TNF-α (50 ng/ml) and then assayed for viability and total cells remaining. Isolated $CD4^+$ cells from both C57 and NOD mice showed identical trends (Fig. 7D). In contrast, $CD8^+$ cells from C57 mice all showed an increase in viability in response to TNF-α; the $CD8^+$ cells from NOD mice manifested cell subset-specific

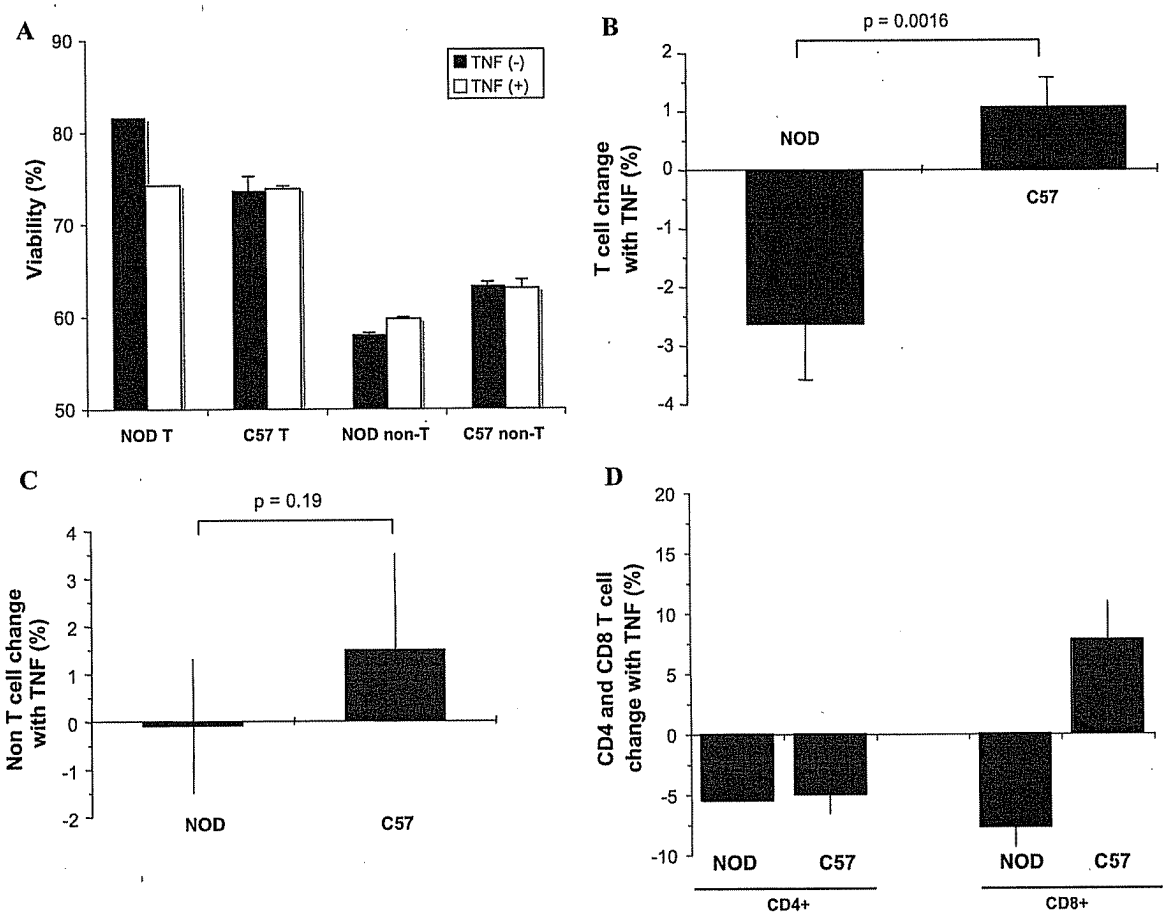

Fig. 6. Increased sensitivity of NOD splenic $CD8^+$ T cells to TNF-α-induced apoptosis. (A) T cells and non-T cells were isolated from splenocyte preparations of NOD and C57 mice, cultured for 24 h with or without TNF-α (50 ng/ml), and assayed for viability by flow cytometric analysis of PI-stained cells (plots of PI fluorescence versus FSC). Data are from 4 and 6 representative experiments. (B and C) Effects of TNF-α on T cell (B) and non-T cell (C) viability as determined in experiments similar to that shown in (A). Data are expressed as the TNF-α-induced change in viability or cell remaining and are means ± S.E.M. of values from 17 and 12 independent experiments, respectively. (D) The T cells isolated from C57 and NOD splenocytes were further fractionated into $CD4^+$ or $CD8^+$ cells and analyzed for TNF-α sensitivity as in (A). Data are from 2 representative experiments performed as triplicates and are expressed as the TNF-α-induced loss in viable cells. Similar experiments were performed a total of 7 times.

death (Fig. 6D). These results indicate that the TNF-α sensitivity of NOD splenic T cells exclusively resides in the $CD8^+$ compartment.

3.6. The sensitivity of NOD splenic T cells to TNF-α-induced apoptosis is correlated with age and extent of insulitis

The penetrance of diabetes in female NOD mice in our animal facility is ~75%. Given that we used late-stage prediabetic mice (>12 weeks of age) for our experiments, some of these mice would be expected not to progress to hyperglycemia. Although such animals (nonresponders) never become diabetic and their pancreatic islets remain free of invasive insulitis, their islets do develop peripheral insulitis, a hallmark of nonprogression. We hypothesized that the splenic T cells of nonresponders would not be sensitive to TNF-α-induced apoptosis. Of the 17 NOD mice whose splenic T cells were analyzed for sensitivity to TNF-α-induced apoptosis (Fig. 6B), 4 animals yielded T cells that showed little or no response to TNF-α. This is the expected number of nonresponders based on a penetrance of 75%. We therefore compared the extent of insulitis in pancreatic tissue of such non-responding mice with that of animals with TNF-α-sensitive T cells.

The islets of NOD mice with T cells sensitive to TNF-α-induced apoptosis typically exhibited pronounced invasive insulitis that resulted in islet destruction (Fig. 7A). In contrast, islets of NOD mice whose T cells did not manifest TNF-α-induced apo-

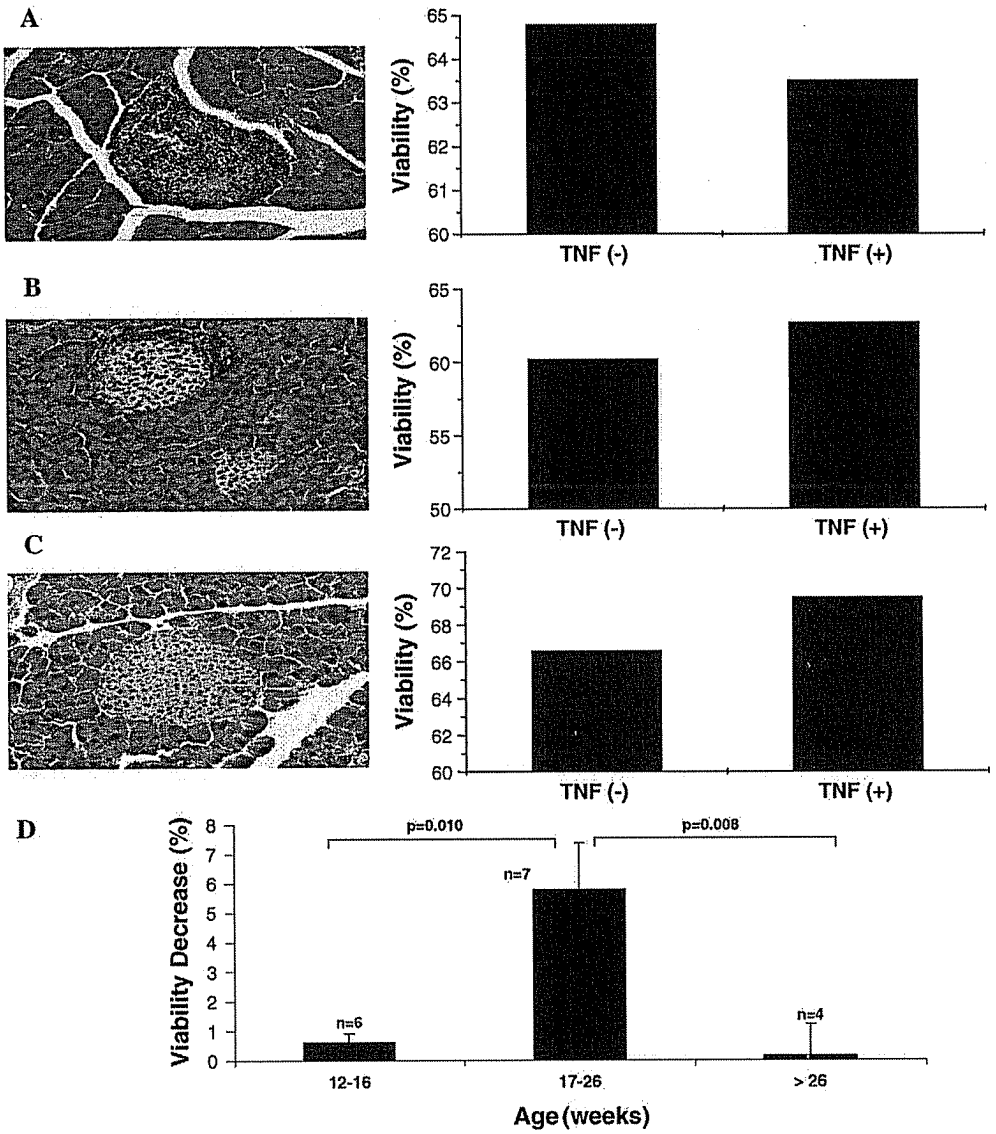

Fig. 7. Correlation of the sensitivity of splenic T cells to TNF-α-induced apoptosis in vitro with age and the extent of insulitis in NOD mice. Freshly prepared splenic T cells from NOD (A and B) and C57 (C) mice were assayed for their sensitivity to TNF-α as in Fig. 7A and the pancreases of the same animals were evaluated by histological staining with hematoxylin–eosin. NOD mice with splenic T cells that were sensitive to TNF-α-induced apoptosis showed marked invasive insulitis and islet destruction (A). In contrast, most of the islets of NOD mice whose splenic T cells did not manifest TNF-α-induced death showed peripheral or no insulitis (B). The islets of control C57 mice, whose T cells did not exhibit TNF-α-induced apoptosis, showed no insulitis (C). (D) The highest TNF-α sensitivity of the NOD T cells occurs during the time period where the NOD are expected to turn diabetic.

ptosis typically showed only peripheral insulitis or no insulitis (Fig. 7B), with only the occasional islet exhibiting invasive insulitis. The islets of C57 control mice were free of insulitis (Fig. 7C).

We also were able to correlate the sensitivity to TNF-α induced apoptosis of the NOD T cells with disease duration. We divided the same group of 17 NOD mice into three sub-groups according to their age at assay time; 12–16 weeks old (group A), 17–26 weeks old (group B), and older then 26 weeks (group C); and calculated the average TNF-α induced decrease in viability for each group (Fig. 7D). The highest TNF-α sensitivity was found for T cells from group B with a decrease in viability of $5.78 \pm 1.60\%$ ($n=7$), as compared to $0.64 \pm 0.27\%$ ($p=0.010$, $n=6$) for group A and $0.16 \pm 1.05\%$ ($p=0.008$, $n=4$) for group C. There was no statistically significant difference between group A and group C ($p=0.35$). These results correlate well with

the age at which the animals are expected to become diabetic.

4. Discussion

This report describes a standardized and reproducible procedure for the purification and culture of splenic lymphoid cell subsets from the mouse. This allows the identification and quantification of lymphocytes in the autoimmune NOD model with alterations in apoptosis sensitivity. With refined methods of harvesting and culturing fresh viable splenocytes, we have identified and quantified a subpopulation of NOD T cells, predominantly of the $CD8^+$ subtype, that undergoes apoptosis upon exposure to a low concentration of TNF-α in vitro. In mature NOD mice that are progressing to disease, these cells constitute 3–7% of the splenic T cell population. T cells from normal control mice, cultured under the same conditions, exhibit a relative increase in viability of ~7% that is likely attributable to the described normal induction of pro-survival signaling through NF-κB after TNF-α exposure (Beg and Baltimore, 1996; Van Antwerp et al., 1996).

We have also identified a subpopulation of splenic non-T cells in NOD mice that undergoes spontaneous apoptosis during culture. These cells are predominantly of B cell origin and constitute ~10% to 15% of the total non-T cell population under our culture conditions. Therefore with refined splenic isolation and culture conditions, at least two distinct subpopulations of lymphoid cells in mature NOD mice possess an altered sensitivity to apoptosis. Non-T cells isolated from individuals with lupus or Sjögren's syndrome also exhibit an increased sensitivity to spontaneous apoptosis in culture; these cells were also shown to comprise mostly B lymphocytes (Emlen et al., 1994; Gross et al., 2000). Patients with systemic sclerosis, a connective tissue disorder characterized by vascular abnormalities and excessive collagen synthesis, similarly possess PBLs with an increased susceptibility to culture-induced death (Stummvoll et al., 2000). Therefore, both spontaneous forms of murine and human autoimmunity have detectable subpopulations of lymphoid cells with altered death set points.

Diverse human and murine autoimmune diseases are characterized by the presence of lymphoid cells in the thymus and periphery with an altered sensitivity to death. This phenotype of disease appears to arise from a common denominator across several autoimmune diseases—a variety of errors in signaling by NF-κB. This transcription factor plays a tightly regulated role that is central to the immune system for the regulation of genes involved in cell fate, resistance to apoptosis and cytokine balance (Baldwin, 1996). An NF-κB signaling defect has been identified in monocytes of humans with Crohn's disease (Hugot et al., 2001; Ogura et al., 2001), an autoimmune condition of the intestine. The enhanced sensitivity of T cells in NOD or NZB mice to TNF-α has also been attributed to defective NF-κB signaling in a death pathway essential for lymphoid development and selection (Valero et al., 2002; Hayashi and Faustman, 2003). Additionally, the widespread introduction of anti-TNF-α therapies, aimed at inhibiting the cytopathogenic effects of TNF-α and NF-κB signaling, instead has uncovered a sizable fraction of patients developing new autoimmune diseases or aggravated forms of diseases such as lupus, multiple sclerosis and even diabetes (van Oosten et al., 1996; Bloom, 2000).

The subpopulations of lymphocytes with an enhanced susceptibility to apoptosis are pathogenic in NOD mice (Rabin et al., 1994; Hayashi and Faustman, 1999; Leslie et al., 1999; Ryu et al., 2001; Wakeland et al., 2001; Kuhtreiber et al., 2003). These cells are thus not merely indirect biomarkers of autoreactivity or by-products of the disease process, but rather contributors to the disease. The accumulating human and murine evidence in autoimmune models shows anti-TNF-α therapies can exacerbate or induce new autoimmune disease in humans. Additionally, low TNF-α activity may predispose to some forms of human autoimmune disease and autoreactive T cells have heightened TNF-α induced apoptosis. The mechanism of TNF-α therapeutic effect appears to be due to the direct T cell death of a highly pathogenic T cell subpopulation and in animal models this direct TNF-α killing hampers disease and prevents transfer of disease with T cells form diabetic hosts (Kodama et al., 2005). This data supports the contention that the TNF-α susceptible T cells are antigen specific and directly related to the disease process. It is possible to achieve permanent disease reversal in end-stage diabetic NOD mice by a two-component therapeutic protocol aimed at re-establishing apoptosis of diseased cell populations in adult mice (Ryu et al., 2001; Kodama et al., 2003). One component of this approach involves the elimination of pathogenic memory T cells by inducing a transient increase in TNF-α levels. Furthermore, brief exposure of splenocytes from diabetic NOD mice to TNF-α in culture markedly reduces the incidence of disease transfer to naïve male NOD cohorts, and the forced expression of TNF-α results in reversion of already diabetic NOD mice to a nondiabetic state (Ryu et al., 2001; Christen and Von Herrath, 2002). The ability to identify and quantify

pathogenic autoreactive cells in vitro would facilitate both research into pathogenesis of diabetes as well as the diagnosis and monitoring of treatment of this disease in both the NOD mouse and humans.

Only select female cohorts of NOD mice comprising about 85% of the colony progress to disease expression characterized by pronounced hyperglycemia and subsequent death. The islets of these cohorts exhibit massive invasive insulitis. The spleens of these animals, leading up to diabetes, have TNF-α sensitive T cell subpopulations. Our biomarker assay also identified splenic T cells did not exhibit enhanced sensitivity to TNF-α-induced apoptosis when disease progression did not occur i.e. aged normoglycemic NOD females. Histological examination of the pancreas of such mice revealed that the islets did not exhibit invasive insulitis, indicating that these animals were TNF-α unresponsive and would not have developed diabetes.

The development of better biomarkers based on cellular defects is important but difficult. It is becoming increasingly clear that the traditional methods of RBC lysis or Ficoll gradients to prepare splenocytes or PBLs can cause extensive damage to the cells. This makes the cells less suitable for functional studies and this is a particular problem in death assays. Similar conclusions have also been reached by the member laboratories of the Alliance for Cellular Signaling (AfCS). Indeed, in a detailed AfCS research report, Hsueh et al. (2002) have defined the essential splenic B cell isolation and culture conditions for meaningful signal transduction studies (Sambrano et al., 2002). This report also emphasizes the requirement of gentle methods to optimize cell viability.

At least two hurdles remain to the adaptation of our biomarker assay to human autoimmune diabetes. First, it will be necessary to sample peripheral blood rather than splenocytes. Given that human blood consists mostly of RBCs, the negative selection procedures that removed T cells or non-T cells from mouse splenocyte preparations are likely to be less effective with human blood as the starting material. Second, unlike NOD mice, humans with autoimmune diabetes are genetically diverse.

Acknowledgements

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Selective death of autoreactive T cells in human autoimmunity by TNF or TNF receptor 2 agonism

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Abbreviations: AnV Annexin V, CTL, cytotoxic T lymphocyte, NOD, non-obese diabetic mouse, PBLs peripheral blood mononuclear cells, PI, propidium iodide, SSC side light scatter, TNF, tumor necrosis factor, TNFR, tumor necrosis factor receptor

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Human autoimmune (AI) diseases are difficult to treat because immunosuppressive drugs are non-specific, produce high levels of adverse effects, and are not based on mechanistic understanding of disease etiology. Destroying the rare autoreactive T lymphocytes causing AI diseases would improve treatment. In animal models, advances in mechanistic understanding have led to successful use of the immunoregulatory cytokine tumor necrosis factor (TNF) to selectively kill autoreactive T cells, thereby hampering disease onset or progression. Here we seek to determine whether TNF, or a less toxic agonist of TNF, can selectively kill autoreactive T cells, but not normal T cells, in fresh specimens of human blood. We isolated highly pure CD4 or CD8 T cells from patients with type 1 diabetes (N=675), other AI diseases, and healthy controls (N=512). Using two cell death assays, we found a subpopulation of CD8, but not CD4, T cells from patients' blood that was vulnerable to TNF or TNFR agonist-induced death. The agonist was a monoclonal antibody that bound TNF receptor 2 (TNFR2), but not TNFR1, which is more ubiquitously expressed. The TNFR2 agonist also exhibited a dose-response killing pattern. In type 1 diabetes, the subpopulation of CD8 T cells susceptible to TNF or TNFR2 agonist-induced death was traced specifically to autoreactive T cells to insulin, a known autoantigen. This shows that autoreactive T cells, although rare, can be selectively destroyed in isolated human blood. Their destruction with TNF or a TNFR2 agonist offers a potentially new targeted and less toxic method of treating or eliminating AI.

TNF/TNFreceptor/human/autoimmunity/apoptosis

In animal models, the immunoregulatory cytokine tumor necrosis factor (TNF) has been successfully used, directly or indirectly, as treatment for several autoimmune diseases (1-4). TNF augmentation also might be beneficial for humans with autoimmunity, based on indirect evidence from genetic and functional studies that suggests possible deficiencies in their TNF levels or signaling pathways. Here we directly test the addition of TNF, or a less toxic agonist of TNF, to human blood specimens, for their capacity to kill only autoreactive T cells from several autoimmune diseases, while sparing normal T cells.

TNF is a vital cytokine in diverse immune responses, including enhanced cell survival or apoptosis (5, 6). TNF achieves these effects via signaling pathways through two membrane receptors, TNFR1 (CD120a; p55/60) and TNFR2 (CD120b; p75/80). TNFR1 is ubiquitously expressed on all T cell populations, the entire lymphoid system, and most other cells. This explains TNF's systemic toxicity when used at high doses in oncology treatment and in diseases with normal TNF basal levels (7, 8). TNFR2, in contrast, is more restrictively expressed, found only on select subpopulations of T cells, endothelial cells, neurons, and other occasional cells (9). Here we take advantage of the apoptotic role of TNF and the differential patterns of TNFR expression to test a targeted method of administering TNF to selectively kill autoreactive T cells. This approach opens the way for a novel and more targeted strategy to treat autoimmunity.

Research over recent decades has been devoted to understanding the TNF receptor pathway and its normal role in T cell survival or in apoptosis to curb growth of activated T cell subpopulations once the response has succeeded. Upon TNF exposure, normal T cells rapidly activate NFκB, a transcription factor that leads to their survival. NFκB in the cytoplasm is part of a larger protein complex that requires both direct and

indirect proteasome processing and ubiquination of inhibitory subunits for successful transport to the nucleus to promote transcription of cell survival genes. When chemicals or mutations block NFκB activation *in vitro*, TNF signaling selectively triggers cell death via apoptosis, especially in activated T cells, which are dependent on this induced pathway (10, 11).

Defects in TNF signaling, most likely in lymphocyte subpopulations, are common across several autoimmune diseases in *both* animals and humans (12-24). The defects, whether genetic and/or functional in origin, affect activation of NFκB. The first described defect was in the autoimmune-prone NOD mouse, a model of spontaneous diabetes and Sjogren's syndrome. In this model, the LMP2 proteasome subunit protein expression decreases as mice progress towards disease. The missing proteasome LMP2 subunit blocks the activation of the NFκB signaling complex, preventing it from entering the nucleus to activate pro-survival genes (16). Proteasome blockage also leads to TNF mRNA instability, thereby lowering levels of TNF translation (17). Remarkably, this same proteasome subunit protein is missing in one form of human autoimmune disease, Sjogren's syndrome (18). Genetic and protein processing defects in the NFκB pathway are also found in humans with lupus, Crohn's, rheumatoid arthritis, and ulcerative colitis (13, 19-21). These findings, taken together, suggest that exogenous TNF prevents entry of unprocessed NFκB into the nucleus, thereby leading to TNF-induced apoptosis of autoreactive T cells.

To confirm TNF's role as a selective killer of potentially autoreactive CD8 T cells in humans, we studied blood specimens from autoimmune patients. We exposed TNF, as well as agonists that mimicked TNF's function, to T cells isolated from the specimens.

One TNF agonist was selective for TNFR1, while others for TNFR2. We sought to answer several questions. Can TNF-induced cell death be observed in a subpopulation of T cells isolated from blood and with enriched detection of rare autoreactive T cells? Is TNF-induced death restricted only to a subpopulation of CD8 cells potentially cytotoxic to their target organs? Can TNF be shown in culture to kill these potentially autoreactive CD8 T cells specific to a given autoantigen? Finally, in pursuit of a less toxic therapeutic approach, we ask whether those autoreactive T cells also can be killed by an agonist for a single TNF receptor, such as TNFR2, which is more limited in cellular expression distribution than TNFR1?

Results

Type 1 diabetics have a subpopulation of T cells that die with TNF exposures: lessons from Ficoll separated blood Ficoll separated PBLs from type 1 diabetic and paired controls were cultured for 12 hours with or without TNF (20 ng/ml). Cells were then evaluated for cell death by flow cytometry and lymphocyte subset specific antibodies (Table 1). An initial analysis of blood samples from 44 paired type 1 diabetes and controls exposed to TNF revealed in diabetic samples a non-significant increase in death of CD3+ T cells. Death was detected with propidium iodide (PI) staining with an anti-CD3 antibody. Assuming that TNF triggers death in only rare autoreactive T cells, we expanded the sample size to 79 diabetics and controls to detect an effect. A greater percentage of diabetics had T cells that again died, but still without reaching significance. To enhance the likelihood of finding a trend, the samples were powered up to 387 diabetic and control blood samples. We found that TNF killed a significantly greater subpopulation of T cells ($p=0.003$) in diabetic samples, although the frequency of TNF-sensitive cells was still small, approximately 1.17% of all CD3+ cells (P-C).

Several limitations emerged from the Ficoll study regarding standardization of blood samples and quantification of T cells sensitive to TNF-triggered death. As the most common method of separating PBLs from massive numbers of RBCs in human blood, this gradient technology produces lymphocytes of poor viability, yield and purity. By the end of isolations, Ficoll preparations typically contain 30-60% dead cells quantified by flow cytometry using forward and side scatter (SI Fig1A). With yields of only 20-40% of starting T cells from fresh blood, large sample sizes are necessary for consistency.

Another limitation is using a single time point of non-synchronized T cell death triggered by TNF, which requires large cell numbers per study condition. A single monitoring time point is a blend of cell loss (dead cells already lysed) and detectable dying cells. Because TNF-triggered cell death occurs in a non-synchronized manner in blood preparations, it is difficult to optimize a single time point for flow analysis and thus standardize viability analysis between different samples (SI Fig1B).

To standardize T cell preparations from freshly drawn blood, we applied non-gradient separation methods. Direct positive selection of magnetically tagged CD4 or CD8 T cells yielded more viable cells (SI Fig1A) that were pure and more representative to the original numbers of starting cells (SI Fig1C). T cell separation methods were further automated using robotic platforms to allow high yields and consistently viable preparations. Fresh samples using solely magnetic separation preparations were >95% viable, >95% pure and achieved >85% yield of the starting cells in blood. This contrasted with typical preparations of Ficoll-isolated PBLs with viability of 30-60%, poor purity, and yields of only 20-40% of starting samples. A series of 256 samples from both diabetics and controls showed reproducibility of separation methods (SI Fig1C). The magnetic separation methods were used thereafter, eliminating the need for large sample sizes because each sample was more representative and highly viable.

TNF Treatment Induces Death of a Subpopulation of CD8 but not CD4 T Cells in Autoimmune Patients. To determine whether freshly isolated preparations of human diabetic T cells were killed with TNF, we studied two types of purified and highly viable subpopulations of CD4 versus CD8 T cells using two different assays.

The first was the LDH assay, which measures cell death and cell proliferation via lactate dehydrogenase, a product in the cytosol of damaged cells. In isolated CD4 T cells, no TNF-induced killing was observed. TNF induced mild proliferation, but no cell death, in both patient and control cells (Fig 1A). No significant differences were observed at all TNF doses, ranging from 0.5-30 ng/ml.

Using the same assay, TNF's effect on CD8 cells was strikingly different. TNF killed diabetic CD8 T cells, but not control CD8 T cells at all TNF doses ($p=0.08$, 0.002, 0.02, 0.01, 0.018 and 0.001). Only 12 pairs of diabetic and control samples were needed to obtain significance (Fig. 1A). As reported in the literature, TNF is known to induce mild T cell proliferation, a finding we too observed, but only in the control samples of purified CD8 T cells, not in CD8 diabetic cells at the higher doses.

As confirmation that TNF kills a subpopulation of diabetic CD8 T cells, an expanded study of 23 pairs of samples from diabetics and controls was examined using the WST-1 assay, which measures cell proliferation directly, but death indirectly. TNF at doses of 1.0 or 2.5 ng/ml induced mild proliferation of control CD8 T cells, but death of diabetic CD8 T cells ($p= 0.0029$, 0.009) (Fig. 1B).

Although the target organs of autoimmune diseases vary, diverse genetic errors in the TNF signaling pathway are a unifying feature that may cause each disease (12-15, 22-24). Purified CD8 T cells from a range of autoimmune patients were studied with the LDH assay. Because other autoimmune patients have normal blood sugars, this study ruled out the impact of high blood sugars, a feature of diabetes that also influences TNF sensitivity. For all autoimmune diseases examined, TNF-sensitive CD8 T cells were detected. TNF killed a subpopulation of CD8 T cells in patients with lupus, psoriasis,

Crohn's, hypothyroidism and multiple sclerosis (SI Fig.2). TNF-induced killing was sufficiently robust that a single autoimmune versus control sample pair could be shown to exhibit a dose-response effect. These findings suggest that TNF-induced killing of a subpopulation of CD8 T cells extends to other AI diseases.

TNFR2 agonist alone kills a subpopulation of CD8 T cells from autoimmune patients. TNF acts by binding to two cell surface receptors, TNFR1 and TNFR2, although the intracellular machinery associated with these receptors is dissimilar. A series of TNFR1 and TNFR2 agonist antibodies were tested on purified CD8 T cells to determine if stimulating only one of the two receptors could kill autoimmune CD8 T cells with greater specificity.

We first examined TNFR1 agonism on purified CD8 T cells from type 1 diabetics compared to controls. Using the LDH assay, TNFR1 agonism caused equally mild CD8 T cell proliferation in diabetic and control CD8 T cells. No significant differences in CD8 T cell responses were found over a range of agonist concentrations. TNFR1 agonist was given to 11 matched pairs at doses of 0.0032, 0.016, 0.08, 0.40, 2 μg/ml. P values were 0.91, 0.74, 0.60, 0.79, 0.65, 0.73, respectively (Fig 2A).

Next we examined three TNFR2 agonists and their ability to induce death in CD8 T cells from diabetic patients, using the LDH assay. TNFR2 agonist clone #1, at all concentrations, and with only a sample size of 8 paired samples, induced CD8 cell death exclusively in diabetic samples (Fig 2B). For the dose range tested, the p values were all significant, at 0.04, 0.02, 0.05, 0.04 and 0.03. Because some TNFR2 antibody agonists are known to be potentiated by addition of TNF, we also incubated the cells with TNF to

observe the impact of bi-receptor stimulation after applying the TNFR2 agonist. Fig 2B*iv* shows that TNF neither potentiated nor inhibited the capacity of this TNFR2 agonist. The killing remained significantly greater in diabetic cells, with p values of 0.01, 0.01, 0.05, 0.04, and 0.01 over the dose range.

Two other TNFR2 agonists were screened for their capacity to induce CD8 T death in diabetic cells, using the LDH assay. Agonist clones #2 and #3 were ineffective at killing significantly more CD8 T cells. They showed no overall differences between diabetics and controls (Fig 2B*ii, iii*) or differences with addition of TNF (Fig 2B *v & vi*). The only exception was that agonist clone #3 stimulated proliferation of control CD8 T cells at the highest TNF concentrations (Fig 2B*vi*).

Since TNFR2 agonist clone #1 effectively and specifically killed a subpopulation of diabetic CD8 T cells—at least as effectively as TNF—we used the WST-1 assay to replicate the finding on a larger sample (n=51 matched pairs). As with the LDH assay, we found a gradual dose-related increase in killing of a subpopulation of diabetic CD8 cells. At agonist doses of 0.1, 0.5 and 1 ug/ml, p values were 0.99, 0.17 and 0.01 (SI Fig 3). Agonist clone #1 also induced slight proliferation of control CD8 cells.

Lastly, TNFR2 agonist clone #1 also killed CD8 T cells from patients with other AI diseases. Not only did this TNFR2 agonist kill CD8 T cells from diabetics, but it also killed CD8 T cells from blood samples from patients with lupus, Graves', psoriasis, and multiple sclerosis (SI Fig 4).

Autoreactive diabetic CD8 T cells to an insulin fragment die with TNFR2 agonism

Autoreactive T cells in diabetics show cytotoxicity against self-peptides correctly

presented through HLA class I alleles(25). For example, peptide-specific autoreactive T cells against the insulin B chain 10-18 have been identified in islet allograft recipients with recurrent autoreactivity by co-incubation of insulin peptide loaded to a class I proteins with freshly isolated lymphocytes (26). With our improved capacity to isolate pure, viable and representative CD8 T cells from fresh blood, we investigated whether long-term diabetics harbor CD8 T cells with reactivity to the insulin peptide fragment in the HLA-A2 (*0201) allele. Those CD8 T cells would be indicative of autoreactive T cells.

A long-term diabetic had detectable autoreactive CD8 T cells with binding to HLA-A2 insulin B10-18 compared to a HLA-A2 non-diabetic control (Fig. 3A). Neither non-diabetic nor diabetic CD8 T cells bound to the irrelevant antigen presented in the control tetramer reagent (tetramer-negative). With more patients and controls using the same methods, somewhat less than 50% of our HLA-A2+ long-term diabetics showed insulin B chain 10-18 autoreactive CD8+ T cells (Fig 3B). An analysis of long-term diabetics with or without insulin tetramer reactive CD8 T cells revealed neither a difference in age of onset of diabetes nor duration of diabetes (Fig. 3C). On average, long-term diabetics with insulin tetramer positive cells were 38.3 +/- 19.4 years and had diabetes for 18.4 +/- 16.6 years. All CD8 cells used for these studies were 95% viable, 95% pure and were representative, with a yield of at least 85% of the starting T cell population.

CD8 autoreactive T cells are thought to represent the defective cells sensitive to TNF-induced death in the earlier WST-1 and LDH assays. To further confirm and refine

the ability of TNFR2 agonists to selectively kill autoreactive T cells, insulin tetramer T cell death was studied in vitro within the highly pure CD8 isolated cells from fresh blood. Fig 4A shows that 6-hour treatment with the R2 agonist of CD8 T cells from a diabetic caused the specific elimination of the insulin autoreactive T cells. The study was expanded to more diabetics who had varying numbers of autoreactive T cells with insulin reactivity to the B chain 10-18 amino acids. Insulin-reactive CD8 T cells treated with brief exposure to the R2 agonist antibody consistently died (Fig 4B). One insulin tetramer positive patient was studied on 4 separate occasions over a three-year time span (Fig 4C). This patient on each visit repeatedly showed the presence of autoreactive CD8 T cells to the insulin + tetramer reagent. The patient's autoreactive cells repeatedly died in culture after low dose exposure to the TNFR2 agonist. In contrast, control cells exposed to TNFR2 agonism demonstrated mild proliferation, no false positive insulin tetramer reactivity, and no death upon exposure to TNFR2 agonists.

Discussion

Destruction of rare autoreactive T cells in autoimmune disease has been an elusive therapeutic goal designed to produce marked benefit over current nonspecific treatments plagued by adverse effects. Here we report that TNF exposure kills a subset of human CD8 T cells from type 1 diabetes and other autoimmune diseases. In contrast, CD4 T cells from type 1 diabetics are resistant to TNF-triggered death. Death of this subpopulation of CD8 T cells is also triggered with a specific agonist for TNFR2 that mimics TNF's actions. But TNFR1 agonists do not trigger cell death. We also show that, in type 1 diabetes, a subpopulation of insulin autoreactive CD8 T cells specific for the HLA class I insulin fragment die with TNFR2 agonism, confirming that the TNF pathway can be used to kill autoreactive T cells.

An abundance of murine models establish the therapeutic benefit for administering TNF or TNF induction to animals with late stage autoimmune disease (1-4). Although many models have been proposed to account for TNF's therapeutic benefit, one candidate mechanism in mouse models is direct autoreactive T cell death (27). This current paper broadly confirms that blood samples taken from humans with diverse autoimmune diseases have similarly sensitive autoreactive CD8 cells populations that selectively undergo apoptosis induced by TNF or a TNF agonist.

The capacity of a TNFR2 agonist to kill autoreactive diabetic CD8 T cells has therapeutic implications for drug safety. Systemic TNF therapy for cancer patients, especially at high doses, can cause toxicity (7, 8). While cancer patients may already have high TNF levels at the onset of therapy, perhaps accounting in part for the added toxicity, the fact remains the therapeutic window for TNF may be narrow. In contrast,

systemic administration of a TNFR2 agonist to baboons, even at high doses, is associated with minimal toxicity (28). This most likely reflects more restricted distribution of the TNFR2 receptor, unlike the body-wide expression of TNFR1. Because we found that autoreactive CD8 T cells die only with TNFR2 stimulation, therapeutic use of TNFR2 agonist without TNFR1 agonism is likely to be less toxic than TNF.

Refinements in our isolation of human T cells made it feasible to study specific subpopulations of T cells with reliability. Traditional methods of separating lymphocytes with Ficoll gradients were labor-intensive and required very large sample sizes to verify pathogenic T cells in long-standing autoimmunity. Gradient separation methods yield only a small fraction of representative T cells of poor viability (29). For the majority of our studies, isolated CD8 T cells were obtained by magnetic cell separation methods, which eliminated traditional gradient separation processes. The yield of CD8 and CD4 T cells from magnetic separations were 95% pure, 95% viable, and represented over 85% of T cells in the starting samples. Our blood separation standardization procedures to retrieve representative T cell populations allowed fewer paired patient and control samples to be obtained, and expedited the identification of T cells sensitive to TNF-triggered cell death. Indeed these refined isolation methods allowed insulin fragment autoreactive T cells to be detected routinely in patients with diabetes of >10 years duration. This last accomplishment was important to establish that the T cells vulnerable to TNF cell death were the rare autoreactive T cells.

Since our findings showing potential benefits of TNF or TNF agonism for treating AI, it seems paradoxical that anti-TNF therapies are a major therapeutic class of drugs currently marketed for AI. TNF antagonists have provided clinical benefit to many autoimmune patients, especially those with rheumatoid arthritis and Crohn's. Yet an expanding body of research in animal models on spontaneous autoimmunity suggests the opposite strategy may be warranted. Furthermore, in humans, several clinical observations deserve mention. First, many Crohn's and rheumatoid arthritis patients never respond to TNF antagonists. Second, long-term treatment with anti-TNF drugs can be accompanied by onset of new or aggravated forms of autoimmunity, sometimes in the form of new autoantibodies, suggesting that, for some AIs, anti-TNF therapy may not be the drug of choice (4). Lastly, some autoimmune diseases like multiple sclerosis worsen when treated with anti-TNF (30-33). Therefore, the new analysis here, combined with previously published animal and human data, argue for the opposite therapeutic strategy for some AI diseases, namely to boost or restore TNF. Activation defects in the transcription factor NFkB, which is part of the TNF signaling pathway, may leave autoreactive T cells more vulnerable to TNF-induced apoptosis. Genetic findings indirectly support polymorphic diversity in this signaling pathway in human AI diseases. Overall, certainly type 1 diabetic patients and perhaps subsets of patients with other AI diseases could benefit from the targeted removal of autoreactive cells with TNF or TNFR2 agonism.

With chronic diseases such as diabetes and other forms of autoimmunity, most therapies have traditionally used non-specific immunosuppression since it was thought

that the rare autoreactive T cells could not be identified, much less selectively killed. A defective, TNF signaling pathway, which leads to cell death, now provides, at least *in vitro,,* a unique opportunity in human AI diseases to kill only autoreactive T cells.

Materials and Methods

Human Subjects: Patients with type 1 diabetes or other autoimmune diseases ranging from lupus, multiple sclerosis, hypothyroidism, celiac disease, Crohn's, Graves', Sjogren's syndrome, and psoriasis were recruited over a 5-year period from the Massachusetts General Hospital with full institutional approval and with informed consent. Further information is available online as supporting information (SI) *Materials and Methods.*

Blood Preparation PBLs were isolated by two major methods, using Ficoll (Table 1, SI Fig. 1) or non-gradient methods using only magnetic beads (all other figures). Further information is available as SI.

Flow Cytometry Studies For most flow cytometry studies, gates were set "open" for inclusion of all cells. The "open gate" included cells of all sizes but excluded cell debris, RBCs, fragmented cells, and apoptotic bodies. The open gate was chosen because cells undergoing cell death, especially by apoptosis, can display changes in light scattering properties. A FACS machine (Becton Dickinson, San Jose, California) was used for the analysis.

TNF receptor antibodies. TNF (Leinco Technologies, St. Louis, MO) and several TNFR1 and TNFR2 antibodies were purchased from the following sources for use on the

magnetically separated T cells. TNFR antibodies were clone MR2-1 (TNFR2) and MR1-2 (TNFR1) (Cell Sciences, Canton, MA), 80M2 (TNFR2) (Cell Sciences HM2022, Canton , MA) (34, 35) and Sigma T1815 (clone 22221.311; Sigma, St Louis, MO) also specific to the TNFR2 receptor. Isotype-specific control antibodies, matched to the antibody agonists, were performed at times to rule out non-specific effects of added immunoglobulin. TNF antibodies also were sometimes tested with the addition of TNF (2.5 ng/ml) to observe if the agonist effect was promoted, enhanced, or reversed.

Detection of cell death For analysis of CD3 cell death within unseparated but Ficoll isolated PBLs, Propidium Iodide (PI) (Oncogene, San Diego, CA) staining was used. CD3 lymphoid subsets were identified by an anti-CD3 antibody (T cells) (Clone UEHT1; BD Pharmingen, San Diego, California). This subset-specific antibody was linked to phycoerythrin.

Two 96 well plate-based assays, WST-1 (Roche Applied Science, Indianapolis, IN) and LDH (Roche Applied Science, Indianapolis, IN) were used to confirm cell death versus viability. The advantage of plate-based assays is the analysis volume of cells is smaller so drug dose-responses can be examined. Plate-based assays also represent accumulated death or proliferative products so a late time point is sometimes adequate without the need to do a time courses on each sample. The disadvantage of the plate-based assays is the relative numbers generated for cell death or viability are related not to absolute numbers of cells proliferating or dying. Further information is available as SI.

Detection of autoreactive CD8 T cells associated with type 1 diabetes. Highly purified CD8 T cells from fresh human blood (within 1.5 hour of venipucture) of greater than

95% purity, 95% viability and 85% yield were stained either fresh or, after 12 hours of culture at 240C followed by 6 hours with TNF or TNFR2 agonist for 6 hours followed by staining with 1 ug/ml of phycoerythrin-labelled class I tetramers (BeckmanCoulter, Fullerton, CA) and CD8 antibodies (Becton Dickinson, NJ). For detection of auto-reactive T cells to insulin, we used tetramers to HLA*0210 insulin beta 10-18 with a fragment of HLVEALYLV (Beckman Coulter #T02001). For negative control tetramers, we used two different reagents: HLA*0201 Her-2/neu with a sequence to KIFGSLAFL (Beckman Coulter #T02001), a breast cancer peptide, and HLA*0201 null without an autoreactive peptide fragment (Beckman Coulter #T01044). Throughout the text we call both of these "empty tetramer reagent" or "negative tetramer reagent". Further information is available on line as SI.

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Figure Legends

Fig 1. TNF treatment of purified human CD4 or CD8 T cells from type 1 diabetics (black bar) compared to controls (shaded bar) for viability versus killing *(A)* TNF treatment of CD4 T cells (n=9 pairs, top) or TNF treatment of CD8 T cells (n=12 pairs, bottom), using the LDH assay. *(B)* TNF treatment of purified human CD8 T cells from larger sample (n=23 pairs) of type 1 diabetics and controls, using the WST-1 assay.

Fig 2. Effect of TNFR1 versus TNFR2 agonist antibodies on death of Type 1 diabetic (dark bar) compared to control (shaded bar) CD8 T cells *(A)* TNFR1 agonist antibody treatment of purified human CD8 T cells from 11 pairs of type 1 diabetics and controls. *(B)* TNFR2 agonist treatment of purified human CD8 T cells without TNF (left column) or with TNF (right column) in type 1 diabetics compared to controls. *i, iv* TNFR2 agonist is clone #1 (n=8 paired samples on left; n= 8 paired samples on right), *ii, v* TNFR2 agonist is clone #2 (n=5 sets of paired samples on left; n=5 paired samples on right), *iii, vi* TNFR2 agonist is clone #3 (n=5 paired samples on left; n=5 paired samples on right). All P values were > 0.05 except for TNFR2 clone #1 with or without TNF.

Fig 3. A subset of insulin autoreactive CD8 T cells can be identified in long term diabetics *(A)* Insulin B10-18 tetramer positive CD8 frequency in a highly pure preparation of CD8 T cells from a long term diabetic (age of onset 13 years, duration of diabetes 8 years) and a paired control. Both patient and control were HLA-A2.1+. Representative two-color dot plots are presented. *(B)* Insulin B10-18 tetramer positive CD8 frequency in long-term type 1 diabetics (N=11) both positive and negative patients and controls. *(C)* Clinical characteristics of HLA-A2 positive type 1 diabetics with and without insulin B10-18 tetramer staining compared to matched controls.

Fig 4. Treatment of insulin autoreactive CD8 T cells with TNFR2 agonist clone #1 kills the pathogenic cells. *(A)* Targeted elimination of insulin B10-18 tetramer+ T cells with a 6 hour treatment with TNFR2 agonist in a long term diabetic compared to matched control T cells *(B)* Insulin B10-18 specific CD8 T cells from type 1 diabetics consistently

decrease in culture when treated with TNFR2 agonist in culture for 6 hours. *(C)* Repeat analysis of the same tetramer insulin positive long term diabetic over a 3-year period repeatedly reveals the insulin autoreactive T cells. Autoreactive CD8 T cells can be repeatedly eliminated with a brief TNFR2 agonist exposure to diabetic cells.

Table 1 A subpopulation of diabetic T cells die with TNF exposures; impact of sample size on death detection

	T Cells exposed to TNF % dead+/-SEM
A. Paired sample size	*n=44*
Type 1 diabetes	2.83+/- 0.98
Controls	2.40+/-0.81
P-C	0.43
Paired P=	**0.446**
B. Paired sample size	*n = 79*
Type 1 diabetes	2.97 +/- 0.22
Controls	2.37 +/- 0.42
P-C	0.60
Paired P=	**0.223**
C. Paired sample size	*n=387*
Type 1 diabetes	3.52 +/- 0.08
Controls	2.35 +/- 0.02
P-C	1.17
Paired P=	**0.003**

Fig. 1

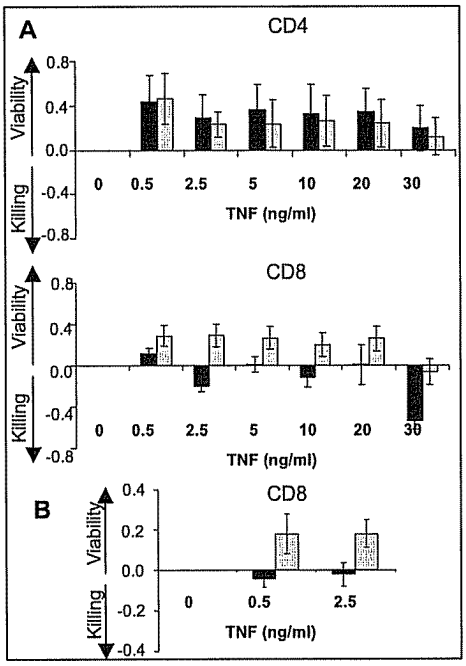

A
CD4
Viability
Killing
0.8
0.4
0.0
-0.4
-0.8
0
0.5
2.5
5
10
20
30
TNF (ng/ml)
CD8
Viability
Killing
0.8
0.4
0.0
-0.4
-0.8
0
0.5
2.5
5
10
20
30
TNF (ng/ml)
B
CD8
Viability
Killing
0.4
0.2
0.0
-0.2
-0.4
0
0.5
2.5
TNF (ng/ml)

Figure 2

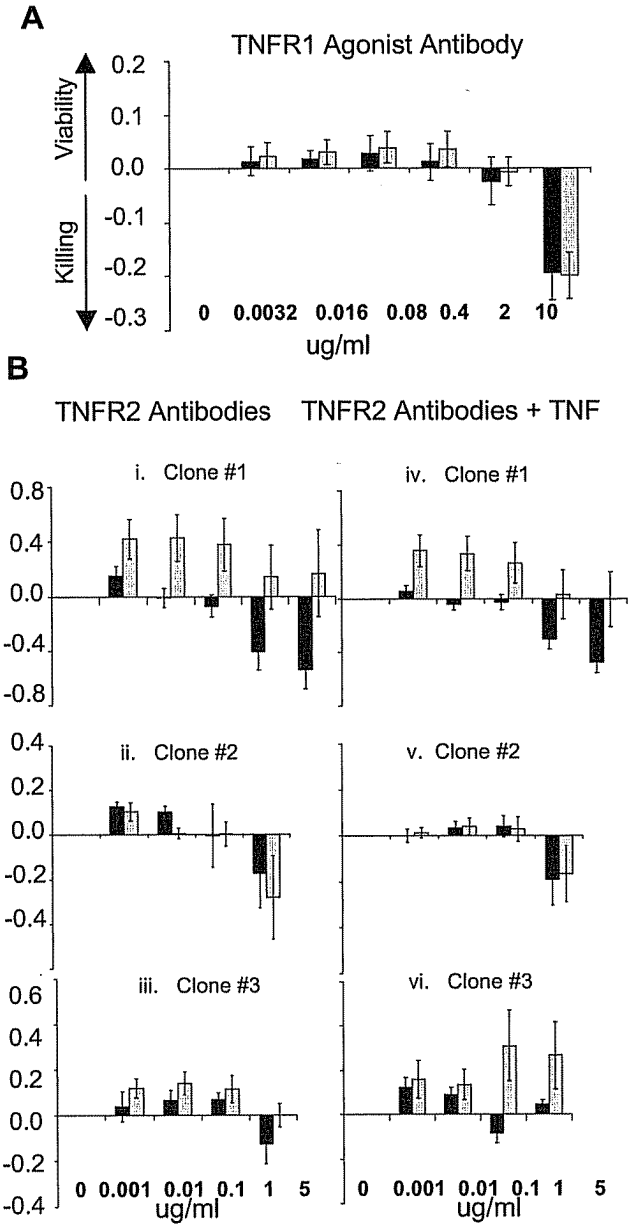
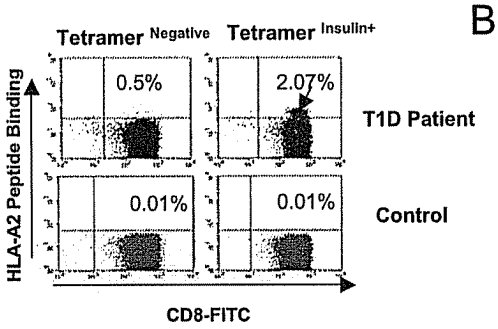
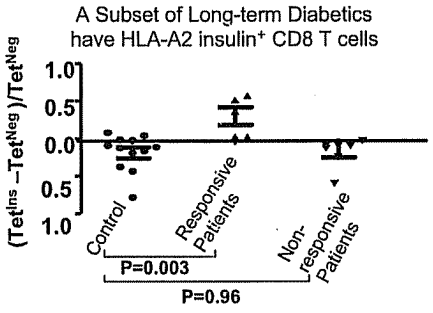

A
TNFR1 Agonist Antibody
Viability
Killing
0.2
0.1
0.0
-0.1
-0.2
-0.3
0 0.0032 0.016 0.08 0.4 2 10
ug/ml
B
TNFR2 Antibodies
TNFR2 Antibodies + TNF
i. Clone #1
iv. Clone #1
ii. Clone #2
v. Clone #2
iii. Clone #3
vi. Clone #3
0 0.001 0.01 0.1 1 5
ug/ml

Figure 3

A



B

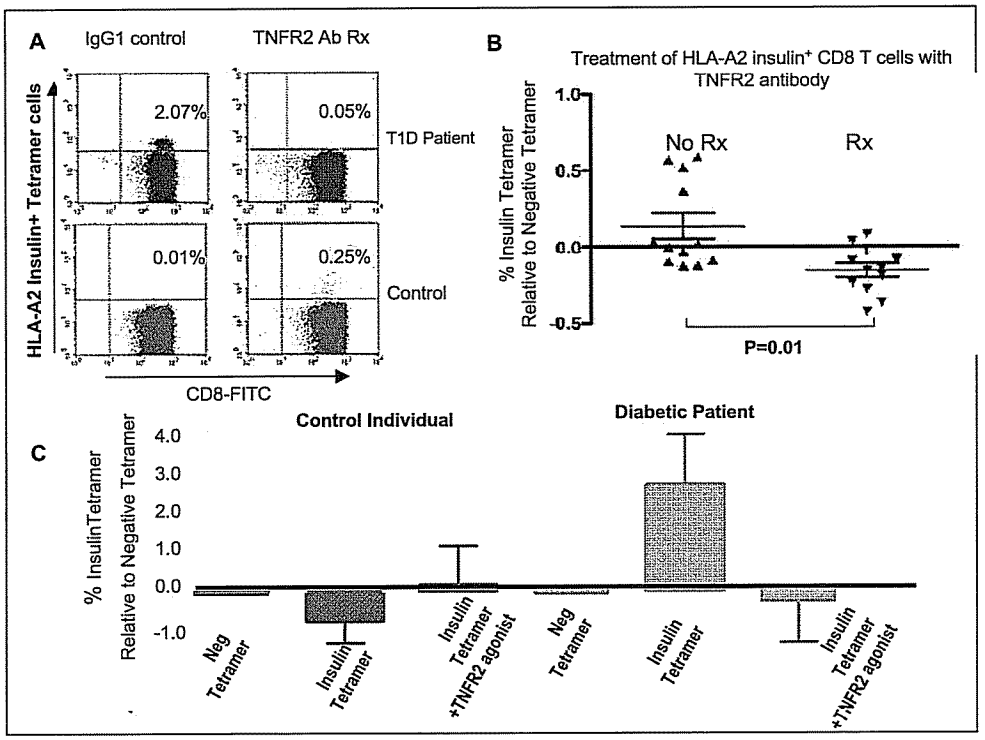


C

Clinical Characteristics of HLA-A2 positives Type I Diabetics and Controls

	N	Age (years) Mean +/- SD	Sex (M/F)	Age of Onset (years) Mean +/- SD	Diabetes Duration (years) Mean +/- SD
Type I Diabetics:					
Insulin tetramer positive	7	38.3 +/- 19.4	5/2	19.9 +/- 8.9	18.4 +/- 16.6
Insulin tetramer negative	4	36.8 +/- 20.0	1/3	17.0 +/- 8.8	19.8 +/- 15.2
Controls	9	38.3 +/- 11.9	5/4	N/A	N/A

Figure 4


A
IgG1 control
TNFR2 Ab Rx
HLA-A2 Insulin+ Tetramer cells
2.07%
0.05%
T1D Patient
0.01%
0.25%
Control
CD8-FITC
B
Treatment of HLA-A2 insulin+ CD8 T cells with TNFR2 antibody
% Insulin Tetramer Relative to Negative Tetramer
1.0
0.5
0.0
-0.5
No Rx
Rx
P=0.01
C
Control Individual
Diabetic Patient
% InsulinTetramer Relative to Negative Tetramer
4.0
3.0
2.0
1.0
0.0
-1.0
Neg Tetramer
Insulin Tetramer
Insulin Tetramer +TNFR2 agonist
Neg Tetramer
Insulin Tetramer
Insulin Tetramer +TNFR2 agonist

SI Materials and Methods

Human Subjects: Patients with type 1 diabetes or other autoimmune diseases ranging from lupus, multiple sclerosis, hypothyroidism, celiac disease, Crohn's, Graves, Sjogren's syndrome, and psoriasis were recruited over a 5-year period from the Massachusetts General Hospital with full institutional approval and with informed consent.

All type 1 diabetic patients were in good health, not in renal failure, had neither received kidney transplants nor systemic immunosuppressive therapy, and had longstanding disease of at least 4-years duration. All other autoimmune patients were on standard therapy regimens for their particular disease. Non-autoimmune controls were screened for autoimmune diseases and had no personal or family history of autoimmunity. Although the first patients and controls studied by the Ficoll method of blood separation were unblinded, all subsequent patient and control blood samples were supplied to laboratory personnel in a blinded fashion.

The design for all experiments was to simultaneously study an autoimmune blood sample paired to a non-autoimmune blood sample taken in parallel on the same day and within 1.5 hours of the first sample. All blood was used fresh and was processed the same day for the various cell death assays of this research project.

After informed consent, all patient and control subjects' blood was drawn into BD Vacutainer™ tubes (BD, Franklin Lakes, NJ) containing acid citrate and dextrose or EDTA Vacutainer tubes (BD, Franklin Lakes, NJ).

Blood Preparation Peripheral blood lymphocytes (PBLs) were isolated by two major methods, using Ficoll (Table 1, Supporting Fig. 1) or non-gradient methods using only magnetic beads (all other figures).

Ficoll Hypaque (Amersham Biotech. Uppsala, Sweden) gradient centrifugation of fresh human blood followed the manufacturer's protocol. Red blood cells (RBCs) were further removed by a 5-minute (on ice) incubation with NH_4Cl solution (PharMLyse (BD), Franklin Lakes, NJ). PBLs were evaluated for viability and then cultured at 5 X 10^5 cells/ml wells for 12 hours. Viability after the Ficoll and NH_4Cl lysis was similar to that reported in the literature, with 40-60% of lymphocytes being viable and the yield representing less than 15% of CD3 T cells in fresh blood. Viability and yield were calculated using flow cytometric methods. The PBLs were cultured in RPMI-1640 media (Gibco, Grand Island, NY) supplemented with 10% heat inactivated bovine serum albumin and antibiotics at 37°C (100 units/ml of Penicillin; 100g/ml Streptomycin). Some PBL wells were treated with human recombinant TNF-α (R & D, Minneapolis, MN) at 20 ng/ml.

All subpopulations of T cells that were separated from white blood cells were either CD4 or CD8 human T cells that were isolated by Dynal magnetic isolation methods (Product Nos 113-33D & 113-31D, respectively, Invitrogen, Carlsbad, CA). This magnetic cell separation method was perfected to the extent that daily isolated CD8 or CD4 human T cells from blood were 95% viable, 95% pure, with yields of over 85% of human CD8 or CD4 T cells per ml of fresh human blood in the starting sample. This method also allowed the T cells after the magnetic process to be free of attached beads and therefore the membranes of the newly isolated pure cells were open for TNF binding.

These cell separation methods were further standardized and automated, using the BioMek platform for uniformity of the cell preparations (Beckman Coulter Fullerton, CA).

Flow Cytometry Studies For most flow cytometry studies, gates were set "open" for inclusion of all cells. The "open gate" included cells of all sizes but excluded cell debris, red blood cells, fragmented cells, and apoptotic bodies. The open gate was chosen because cells undergoing cell death, especially by apoptosis, can display changes in light scattering properties. A FACS machine (Becton Dickinson, San Jose, California) was used for the analysis.

TNF receptor antibodies. TNF (Leinco Technologies, St. Louis, MO) and a variety of TNFR1 and TNFR2 antibodies were purchased from the following sources for use on the magnetically separated T cells. The TNFR antibodies used were clone MR2-1 (TNFR2) and MR1-2 (TNFR1) (Cell Sciences, Canton, MA), 80M2 (TNFR2) (Cell Sciences HM2022, Canton , MA) (1) (2) specific to TNFR2 and Sigma T1815 (clone 22221.311; Sigma, St Louis, MO) - specific to the TNFR2 receptor. Isotype-specific control antibodies, matched to the antibody agonists, were performed at times to rule out non-specific effects of added immunoglobulin. TNF antibodies also were sometimes tested with the addition of TNF (at concentrations of 2.5 ng/ml) to observe if the agonist antibody effect was promoted, enhanced, or reversed.

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Two 96 well plate based assays, WST-1 (Roche Applied Science, Indianapolis, IN) and LDH (Roche Applied Science, Indianapolis, IN) were used to confirm cell death versus viability. The advantage of plate-based assays is the analysis volume of cells is smaller so drug dose responses can be examined. Plate-based assays also represent accumulated death or proliferative products so a late time point is sometimes adequate without the need to do a time courses on each sample. The disadvantage of the plate-based assays is the relative numbers generated for cell death or viability are related not to absolute numbers of cells proliferating or dying.

The lactate dehydrogenase (LDH) released into cell cultures is an index of cytotoxicity and evaluates the permeability of the cell membrane after cell death. Human peripheral blood CD8+ T cells were isolated using magnetic beads Dynal Beads (see above) and then plated into 96 well U-bottom plates at a concentration of 100,000 cells/well. After an incubation of 48hr with different concentrations of TNF, TNFR1 and TNFR2 antibody, the culture supernatants were measured using the Roche Cytotoxicity Colorimetric Assay Kit, as known as the LDH assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Experiments were examined in triplicate. The absorbance was determined by Beckman Coulter DTX 880 Spectrophotometer (Beckman Coulter, Fullerton, CA) at wavelength 492nm. Test

medium was used as background control. The maximum release of LDH was measured by adding 1% Triton X-100 to the cells. The absorbance value was then analyzed to determine cell proliferation or death compared to control wells using following equation: (TNF Treated – Untreated) / (High Control with Maximum LDH release – untreated).

The WST-1 assay is a cell proliferation assay that indirectly measures cell death. For WST-1 experiments, the isolated CD8 or CD4 T cells were plated into 96 well U-bottom plate with a cell concentration 100,000 cells/well. Cells were cultured overnight at 26°C in RPMI media with 1% heat inactivated fetal calf serum. In the morning, the cells were treated with TNF or TNF receptor agonism for 1 hr. After the 1 hour exposure to TNF or TNF agonism, the WST-1 reagent (Roche Applied Science, Indianapolis, IN), a tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate) was added according to the manufactures instructions. The cleavage of WST-1 to formazan by metabolically active cells was quantified by Beckman Coulters DTX 880 Spectrophotometer (Beckman Coulter, Fullerton, CA) at a wavelength 405nm. Each experiment was performed in triplicate. Test medium was used as background control. The cells treated with various doses of ligand and data are presented as a percentage of proliferation compared to the untreated cells using the following equation: (TNF or TNFR2 antibody treated – untreated)/ untreated).

Throughout this text we use the word viability and proliferation interchangeably and the word death and killing interchangeably. It should be acknowledged that the LDH assay can measure both cell proliferation and death but the WST-1 assay is a cell proliferation

reagent that indirectly measure death by the loss of proliferation or cleavage of the WST-1 reagent.

Detection of autoreactive CD8 T cells associated with type 1 diabetes. Highly purified CD8 T cells from fresh human blood (within 1.5 hour of venipucture) of greater than 95% purity, 95% viability and 85% yield were stained either fresh or, after 12 hours of culture at 24°C followed by 6 hours with TNF or TNFR2 agonist and then stained with 1 ug/ml of phycoerythrin-labelled class I tetramers (Beckman Coulter, Fullerton, CA) and CD8 antibodies (Becton Dickinson, NJ). For detection of auto-reactive T cells to insulin, we used tetramers to HLA*0210 insulin beta 10-18 with a fragment of HLVEALYLV (Beckman Coulter #T02001). For negative control tetramers, we used two different reagents: HLA*0201 Her-2/neu with a sequence to KIFGSLAFL (Beckman Coulter #T02001), a breast cancer peptide, and HLA*0201 null without a non-specific peptide fragment (Beckman Coulter #T01044). Throughout the text we call both of these "empty tetramer reagent" or "negative tetramer reagent".

All cells were stained in the dark for 30 minutes and then kept on ice until flow cytometry on fresh non-expanded cells. Cells were washed twice in Hanks with 2% heat inactivated bovine serum after the tetramer staining. On average 100,000 highly pure CD8 T cells were analyzed to ensure clear data points on the Becton Dickinson FACSCalibur using the CellQuest acquisition program. If cells were to be treated with TNFR2 antibody the cells were exposure to 1ug/ml of clone MR2-1 at a concentration of 200,000-400,000 purified CD8 T cells in 96 wells plates for 6 hours at 37°C after the 12-hour overnight incubation at 28°C. All cells for tetramer staining were never frozen,

cultured nor expanded prior to study. For calculations of insulin tetramer + cells or the elimination of these cells with TNFR2 agonism the following equations were utilized, $(Tetramer^{Insulin} - Tetramer^{Negative})/Tetramer^{Negative}$

Statistical Analysis. Because of the well-described analysis day effect of flow cytometric data or T cell assays, every patient was simultaneously studied with one paired random non-diabetic on the same day. The differences between results in diabetic patients and controls are presented as repeated measure paired t-test by controlling for the experiment running date. Pearson correlation was used to evaluate the relationship of percent cell death in diabetes patients with the disease duration and the age at disease onset. The differences between the effects of exposures were compared separately in patients and controls using the t-test. We considered two-sided p value 0.05 as significant without controlling for multiple comparisons.

SI Figure Legends

SI Fig 1. Comparison of viability of lymphocytes isolated by different separation methods and time course of TNF killing with flow cytometric methods *(A)* Ficoll separated white blood cells (WBCs) from human blood show high death after isolation from blood (left histogram) with 52% of dead cells compared to highly viable T cells using magnetic separation methods (right, histogram), with only 0.5% dead cells. Death measured by flow cytometry using forward scatter (FSC) versus side scatter (SSC). *(B)* Flow cytometry measured death of diabetic compared to control CD8 T cells using flow cytometry counting of dead cells (top) to total cells remaining (bottom) over a time course. *(C)* CD8 T cells separated from fresh human blood by direct magnetic separations consistently have high viability and purity (n=256 blood samples).

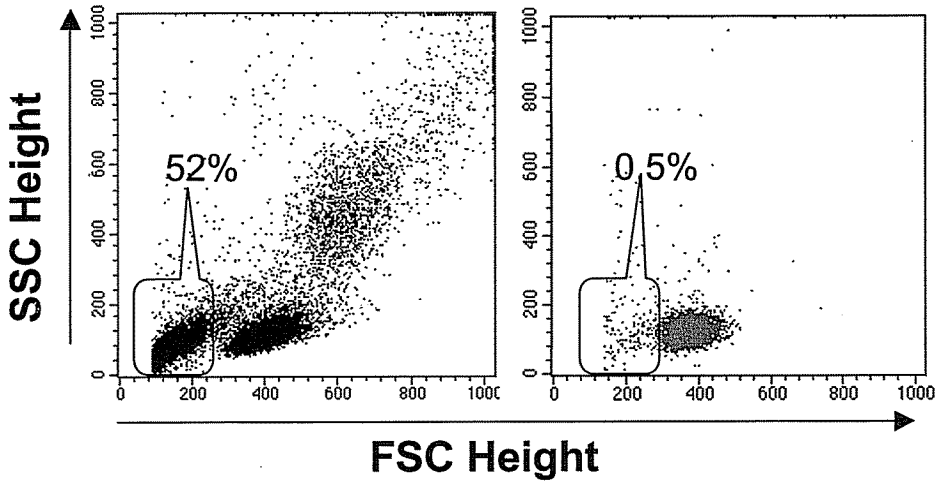
SI Fig 2. TNF treatment of purified human CD8 T cells from autoimmune patients (black bar) compared to controls (shaded bar) for viability versus killing in patients with *(A)* Type 1 diabetes *(B)* Lupus *(C)* Psoriasis *(D)* Crohn's *(E)* Hypothryoidism *(F)* Multiple sclerosis. Each figure represents one patient compared to one control. The WST-1 assay was used for all patients except (D), for which the LDH assay was used.

SI Fig 3. TNFR2 agonist antibody (Clone #1) on death of Type 1 diabetic (dark bar) compared to control (shaded bar) CD8 T cells using the WST-1 assay (n=51 paired samples). As in other assays, the WST-1 assay shows mild proliferation of control CD8 T cells and killing of diabetic CD8 T cells when exposed to TNFR2 agonist antibody Clone #1. Data represents 51 paired type 1 diabetic and control samples. TNFR2 agonist clone #1 at concentrations of 0.1, 0.5, 1 μg/ml correspond to p values of 0.99, 0.17, 0.01.

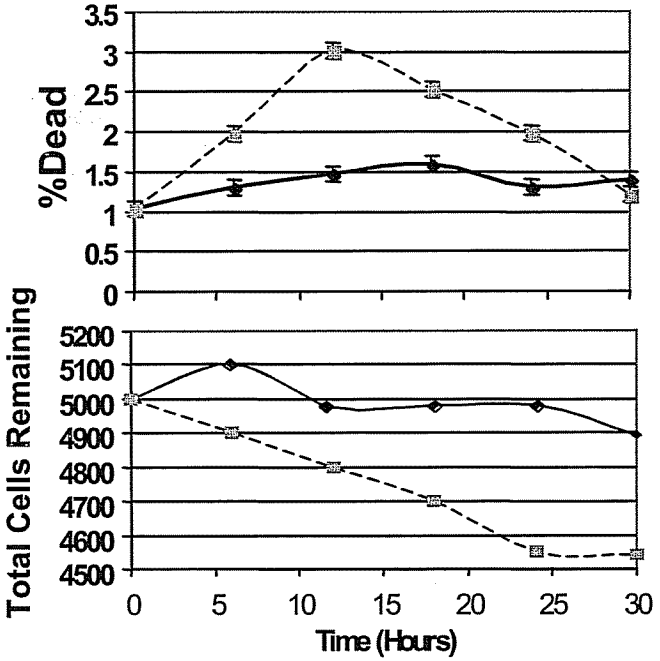
SI Fig 4. TNFR2 agonist (Clone #1) treatment of purified human CD8 T cells from diverse autoimmune patients (black bar) compared to controls (shaded bar) for viability versus killing. *(A)* Type 1 diabetes *(B)* Lupus *(C)* Graves *(D)* Psoriasis *(E)* Multiple Sclerosis Each figure represents one patient compared to one control.

SI Fig1

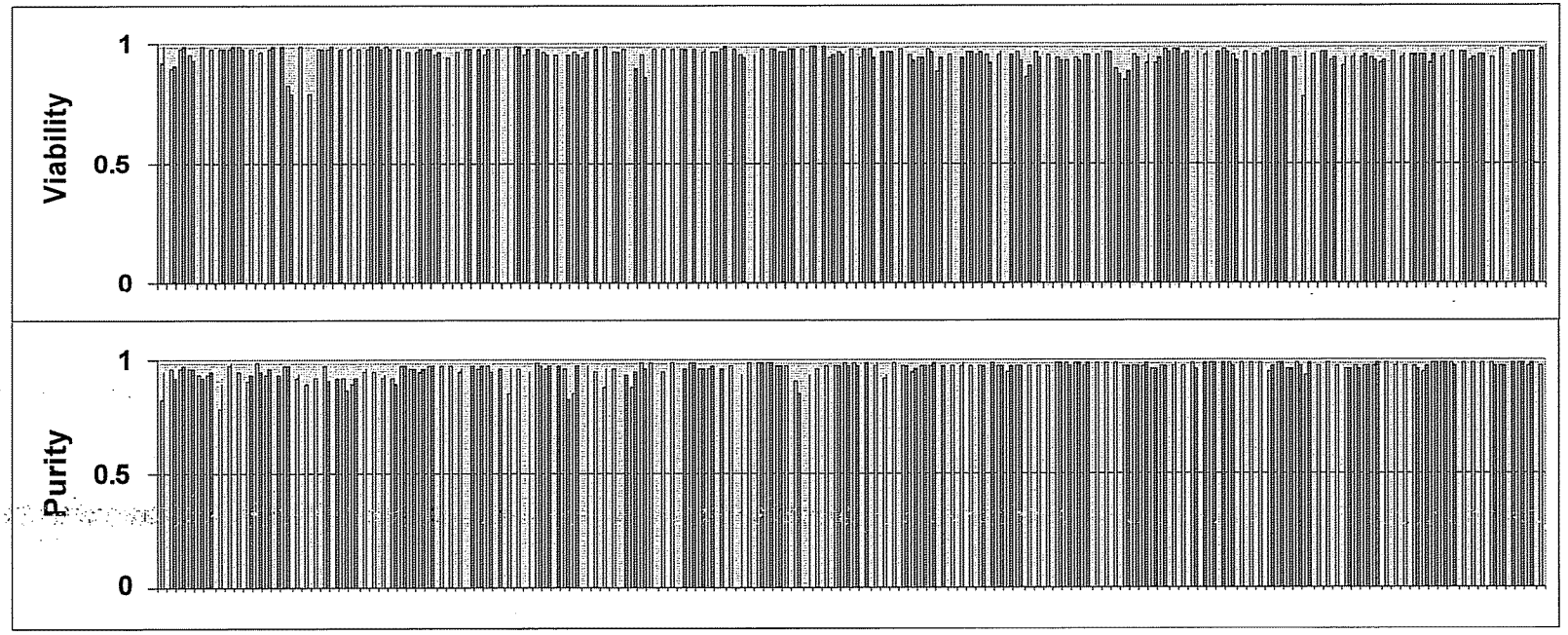
A



B

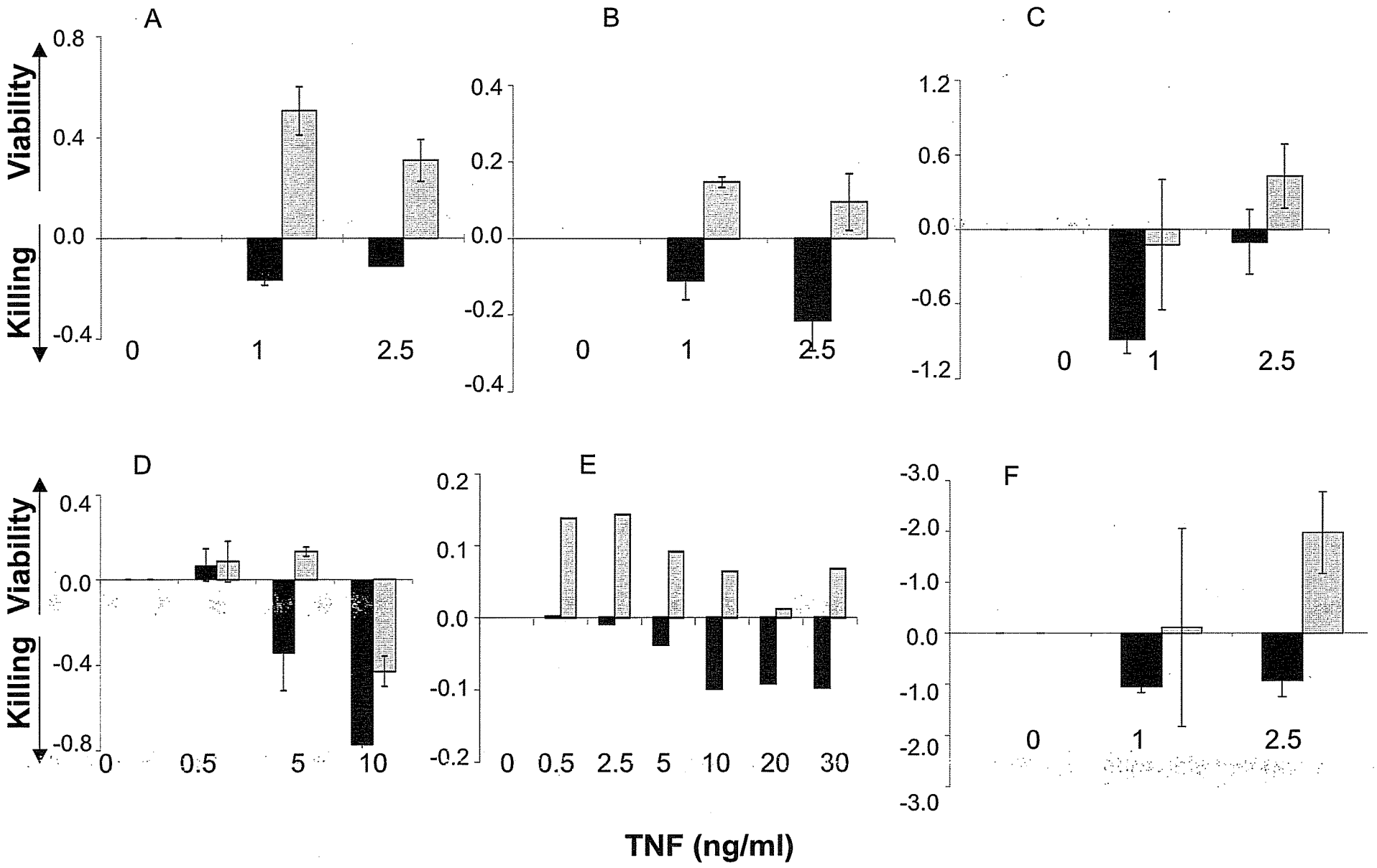


C

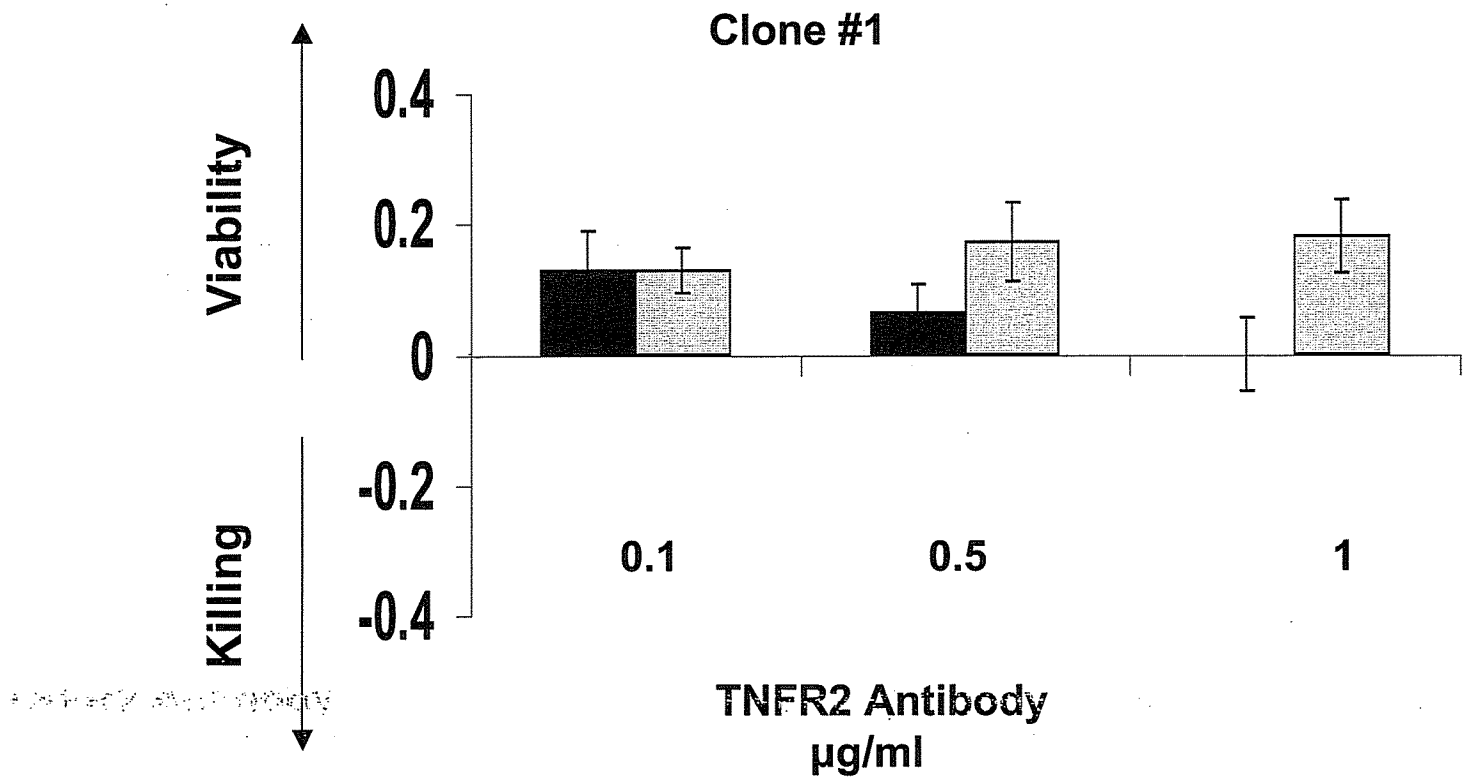


CD8 T cell Viability and Purity Per Individual Sample

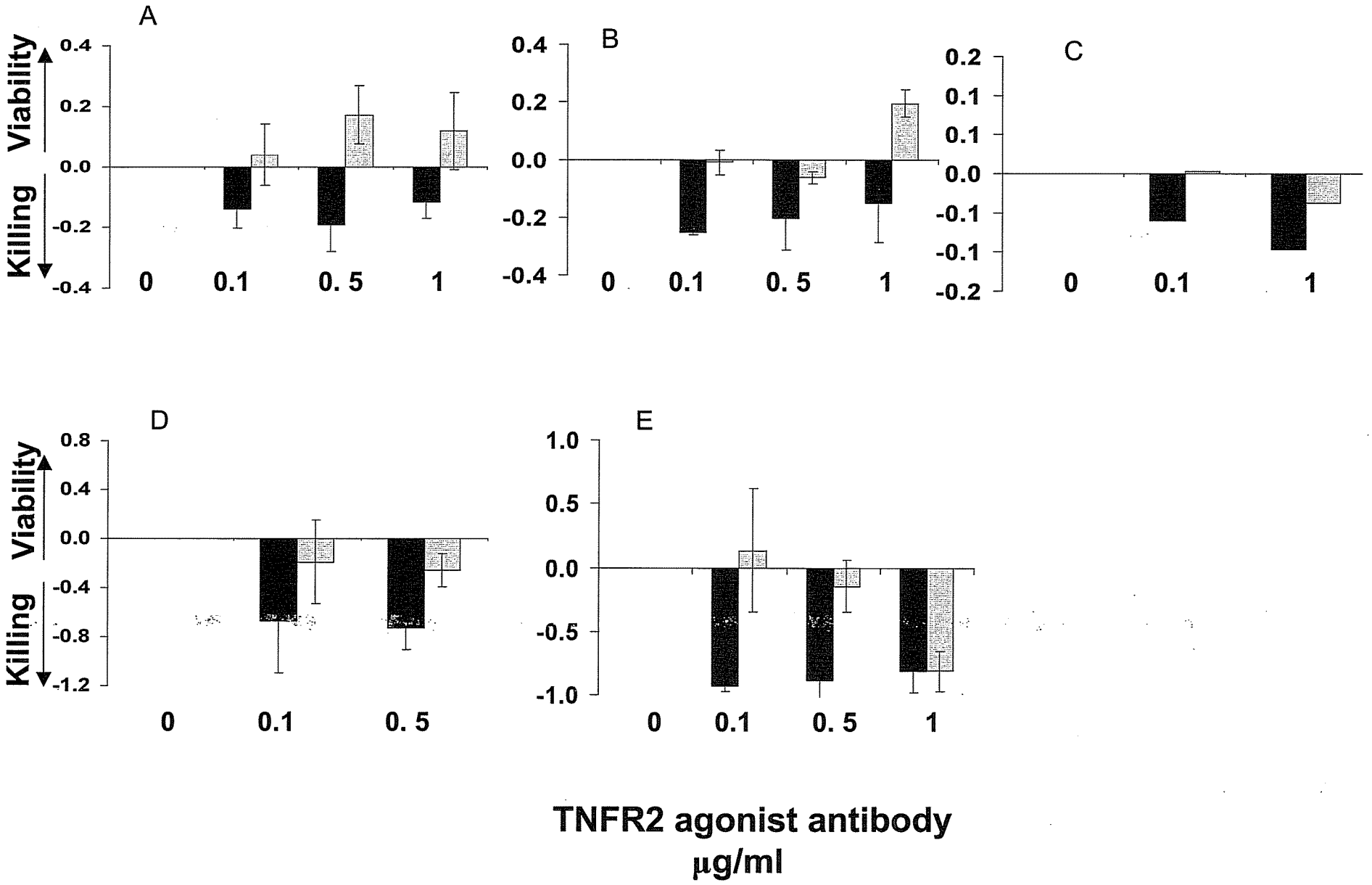
SI Fig 2


A
B
C
D
E
F
Viability
Killing
0.8
0.4
0.0
-0.4
0
1
2.5
0.4
0.2
0.0
-0.2
-0.4
1.2
0.6
0.0
-0.6
-1.2
0.4
0.0
-0.4
-0.8
0
0.5
5
10
0.2
0.1
0.0
-0.1
-0.2
0
0.5
2.5
5
10
20
30
-3.0
-2.0
-1.0
0.0
-1.0
-2.0
-3.0
TNF (ng/ml)

SI Fig 3


Clone #1
Viability
Killing
0.4
0.2
0
-0.2
-0.4
0.1
0.5
1
TNFR2 Antibody
µg/ml

SI Fig 4


A
B
C
D
E
Viability
Killing
0.4
0.2
0.0
-0.2
-0.4
0.1
0. 5
1
0.8
-0.8
-1.2
0.5
-0.5
-1.0
TNFR2 agonist antibody
µg/ml